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(54) Title: A GENE SWITCH COMPRISING AN ECDYSONE RECEPTOR			
(57) Abstract The invention relates to an insect steroid receptor protein which is capable of acting as a gene switch which is responsive to a chemical inducer enabling external control of the gene.			

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A gene switch comprising an ecdysone receptor

The present invention relates to the identification and characterisation of insect steroid receptors from the Lepidoptera species *Heliothis virescens*, and the nucleic acid encoding therefor. The present invention also relates to the use of such receptors, and such nucleic acid, particularly, but not exclusively, in screening methods, and gene switches. By gene switch we mean a gene sequence which is responsive to an applied exogenous chemical inducer enabling external control of expression of the gene controlled by said gene sequence.

Lipophilic hormones such as steroids induce changes in gene expression to elicit profound effects on growth, cellular differentiation, and homeostasis. These hormones recognise intracellular receptors that share a common modular structure consisting of three main functional domains: a variable amino terminal region that contains a transactivation domain, a DNA binding domain, and a ligand binding domain on the carboxyl side of the molecule. The DNA binding domain contains nine invariant cysteines, eight of which are involved in zinc coordination to form a two-finger structure. In the nucleus the hormone-receptor complex binds to specific enhancer-like sequences called hormone response elements (HREs) to modulate transcription of target genes.

The field of insect steroid research has undergone a revolution in the last three years as a result of the cloning and preliminary characterisation of the first steroid receptor member genes. These developments suggest the time is ripe to try to use this knowledge to improve our tools in the constant fight against insect pests. Most of the research carried out on the molecular biology of the steroid receptor superfamily has been on *Drosophila melanogaster* (Diptera), see for example International Patent Publication No WO91/13167, with some in *Manduca* and *Galleria* (Lepidoptera).

It has been three decades since 20-hydroxyecdysone was first isolated and shown to be involved in the regulation of development of insects. Since then work has been carried out to try to understand the pathway by which this small hydrophobic molecule regulates a number of activities. By the early 1970s, through the studies of Clever and Ashburner, it was clear that at least in the salivary glands of third instar *Drosophila* larvae, the application of ecdysone lead to the reproducible activation of over a hundred genes. The ecdysone receptor in this pathway is involved in the regulation of two classes of genes: a small class (early genes) which are induced by the ecdysone receptor and a large class (late genes) which are repressed by the ecdysone receptor. The early class of genes are thought to have two functions reciprocal to those of the ecdysone receptor; the repression of the early transcripts and the induction of late gene transcription. Members of the early genes so far isolated and characterised belong to the class of molecules with characteristics similar to known

transcription factors. They are thus predicted to behave as expected by the model of ecdysone action (Ashburner, 1991). More recently, the early genes E74 and E75 have been shown to bind both types of ecdysone inducible genes (Thummel et al., 1990; Segraves and Hogness, 1991), thus supporting their proposed dual activities. It should be noted however, that the
5 activation of a hierarchy of genes is not limited to third instar larvae salivary glands, but that the response to the ecdysone peak at the end of larval life is observed in many other tissues, such as the imaginal disks (i.e. those tissues which metamorphose to adult structures) and other larval tissues which histolyse at the end of larval life (eg. larval fat body). The model for ecdysone action as deduced by studying the third instar chromosome puffing may not apply
10 to the activation of ecdysone regulated genes in adults. In other words, the requirement for other factors in addition to the active ecdysone receptor must be satisfied for correct developmental expression (e.g. the *Drosophila* yolk protein gene expression in adults is under control of doublesex, the last gene in the sex determination gene hierarchy).

The ecdysone receptor and the early gene E75 belong to the steroid receptor
15 superfamily. Other *Drosophila* genes, including ultraspiracle, tailless, sevenup and FTZ-F1, also belong to this family. However, of all these genes only the ecdysone receptor is known to have a ligand, and thus the others are known as orphan receptors. Interestingly, despite the ultraspiracle protein ligand binding region sharing 49% identity with the vertebrate retinoic X receptor (RXR) ligand binding region (Oro et al., 1990), they do not share the
20 same ligand (i.e. the RXR ligand is 9-cis retinoic acid) (Heymann et al., 1992 and Mangelsdorf et al., 1992). All the *Drosophila* genes mentioned are involved in development, ultraspiracle for example, is required for embryonic and larval abdominal development. The protein products of these genes all fit the main features of the steroid receptor superfamily (Evans, 1988; Green and Chambon, 1988, Beato, 1989) i.e. they have a variable N terminus
25 region involved in ligand independent transactivation (Domains A and B), a highly conserved 66-68 amino acid region which is responsible for the binding of DNA at specific sites (Domain C), a hinge region thought to contain a nuclear translocation signal (Domain D), and a well conserved region containing the ligand binding region, transactivation sequences and the dimerisation phase (Domain E). The last region, domain F, is also very variable and
30 its function is unknown.

Steroid receptor action has been elucidated in considerable detail in vertebrate systems at both the cellular and molecular levels. In the absence of ligand, the receptor molecule resides in the cytoplasm where it is bound by Hsp90, Hsp70, and p59 to form the inactive complex (Evans, 1988). Upon binding of the ligand molecule by the receptor a conformational
35 change takes place which releases the Hsp90, Hsp70 and p59 molecules, while exposing the nuclear translocation signals in the receptor. The ligand dependent conformational change is seen in the ligand binding domain of both progesterone and retinoic acid receptors (Allan et

al., 1992a). This conformational change has been further characterised in the progesterone receptor and was found to be indispensable for gene transactivation (Allan et al., 1992b). Once inside the nucleus the receptor dimer binds to the receptor responsive element at a specific site on the DNA resulting in the activation or repression of a target gene. The receptor responsive elements usually consist of degenerate direct repeats, with a spacer
5 between 1 and 5 nucleotides, which are bound by a receptor dimer through the DNA binding region (Domain C).

Whereas some steroid hormone receptors are active as homodimers others act as heterodimers. For example, in vertebrates, the retinoic acid receptor (RAR) forms
10 heterodimers with the retinoic X receptor (RXR). RXR can also form heterodimers with the thyroid receptor, vitamin D receptor (Yu et al., 1991; Leid et al., 1992) and peroxisome activator receptor (Kliwer et al., 1992). Functionally the main difference between homodimers and heterodimers is increased specificity of binding to specific response elements. This indicates that different pathways can be linked, co-ordinated and modulated,
15 and more importantly this observation begins to explain the molecular basis of the pleotropic activity of retinoic acid in vertebrate development (Leid et al., 1992b). Similarly, the *Drosophila* ultraspiracle gene product was recently shown to be capable of forming heterodimers with retinoic acid, thyroid, vitamin D and peroxisome activator receptors and to stimulate the binding of these receptors to their target responsive elements (Yao et al., 1993).
20 More significantly, the ultraspiracle gene product has also been shown to form heterodimers with the ecdysone receptor, resulting in cooperative binding to the ecdysone response element and capable of rendering mammalian cells ecdysone responsive (Yao et al., 1992). The latter is of importance since transactivation of the ecdysone gene alone in mammalian cells fails to elicit an ecdysone response (Koelle et al., 1991), therefore suggesting that the ultraspiracle gene product is an integral component of a functional ecdysone receptor (Yao et al., 1992).
25 It is possible that the ultraspiracle product competes with other steroid receptors or factors to form heterodimers with the ecdysone receptor. Moreover it remains to be investigated if ultraspiracle is expressed in all tissues of the *Drosophila* larvae. Despite ultraspiracle being necessary to produce a functional ecdysone receptor, the mechanism by which this activation
30 takes place is as yet undetermined.

We have now isolated and characterised the ecdysone steroid receptor from *Heliothis virescens* (hereinafter HEcR). We have found that surprisingly unlike the *Drosophila* ecdysone steroid receptor (hereinafter DEcR), in reports to-date, HEcR can be induced by known non-steroidal inducers. It will be appreciated that this provides many advantages for
35 the system.

Steroids are difficult and expensive to make. In addition, the use of a non-steroid as the inducer allows the system to be used in agrochemical and pharmaceutical applications, not

least because it avoids application of a steroid which is already present in insects and/or mammals. For example, it would not be feasible to use a gene switch in a mammalian cell which was induced by a naturally occurring steroidal inducer. It will also be appreciated that for environmental reasons it is advantageous to avoid the use of steroids as inducers.

5 According to one aspect of the present invention there is provided DNA having the sequence shown in Seq ID No. 2, wherein Seq ID No 2 gives the sequence for the HEcR.

 According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 2, which encodes for the HEcR ligand binding domain.

10 According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 2, which encodes for the HEcR DNA binding domain.

 According to yet another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 2, which encodes for the HEcR
15 transactivation domain.

 According to a further aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 2, which encodes for the HEcR hinge domain.

 According to a still further aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 2, which encodes for the HEcR carboxy
20 terminal region.

 According to one aspect of the present invention there is provided DNA having the sequence shown in Seq ID No. 3, wherein Seq ID No 3 gives the sequence for the HEcR.

 According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 3, which encodes for the HEcR ligand binding
25 domain.

 According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 3, which encodes for the HEcR DNA binding domain.

 According to yet another aspect of the present invention there is provided DNA
30 having part of the sequence shown in Seq ID No. 3, which encodes for the HEcR transactivation domain.

 According to a further aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 3, which encodes for the HEcR hinge domain.

 According to a still further aspect of the present invention there is provided DNA
35 having part of the sequence shown in Seq ID No. 3, which encodes for the HEcR carboxy terminal region.

According to one aspect of the present invention there is provided DNA having the sequence shown in Seq ID No. 4, wherein Seq ID No 4 gives the sequence for the HEcR.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 4, which encodes for the HEcR ligand binding domain.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 4, which encodes for the HEcR DNA binding domain.

According to yet another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 4, which encodes for the HEcR transactivation domain.

According to a further aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 4, which encodes for the HEcR hinge domain.

According to a still further aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 4, which encodes for the HEcR carboxy terminal region.

As mentioned above, steroid receptors are eukaryotic transcriptional regulatory factors which, in response to the binding of the steroid hormone, are believed to bind to specific DNA elements and activate transcription. The steroid receptor can be divided into six regions, designated A to F, using alignment techniques based on shared homology with other members of the steroid hormone receptor superfamily. Krust et al identified two main regions in the receptor, C and E. Region C is hydrophilic and is unusual in its high content in cysteine, lysine and arginine. It corresponds to a DNA-binding domain, sometimes referred to as the "zinc finger". It is the DNA binding domain which binds to the upstream DNA of the responsive gene. Such upstream DNA is known as the hormone response element or HRE for short. Region E is hydrophobic and is identified as the hormone (or ligand) binding domain. Region E can be further subdivided into regions E1, E2 and E3.

The region D, which separates domains C and E is highly hydrophobic and is flexible. It is believed that communication between domains E and C involves direct contact between them through region D, which provides a hinge between the two domains. Region D is therefore referred to as the hinge domain.

The mechanism of the receptor appears to require it to interact with some element(s) of the transcription machinery over and above its interactions with the hormone and the hormone response element. N-terminal regions A and B perform such a function and are jointly known as the transactivation domain. The carboxy-terminal region is designated F.

The domain boundaries of the HEcR can be defined as follows:

DOMAIN	INTERVALS	
	base pairs	amino acids
Transactivating (A/B)	114-600	1-162
DNA Binding (C)	601-798	163-228
Hinge (D)	799-1091	229-326
Ligand Binding (E)	1092-1757	327-545
C-Terminal End (F)	1758-1844	546-577

The DNA binding domain is very well defined and is 66 amino acids long, thus providing good boundaries. The above intervals have been defined using the multiple alignment for the ecdysone receptors (Figure 5).

The present invention also includes DNA which shows homology to the sequences of the present invention. Typically homology is shown when 60% or more of the nucleotides are common, more typically 65%, preferably 70%, more preferably 75%, even more preferably 80% or 85%, especially preferred are 90%, 95%, 98% or 99% or more homology.

The present invention also includes DNA which hybridises to the DNA of the present invention and which codes for at least part of the *Heliothis* ecdysone receptor transactivation domain, DNA binding domain, hinge domain, ligand binding domain and/or carboxy terminal region. Preferably such hybridisation occurs at, or between, low and high stringency conditions. In general terms, low stringency conditions can be defined as 3 x SCC at about ambient temperature to about 65°C, and high stringency conditions as 0.1 x SSC at about 65°C. SSC is the name of a buffer of 0.15M NaCl, 0.015M trisodium citrate. 3 x SSC is three times as strong as SSC and so on.

The present invention further includes DNA which is degenerate as a result of the genetic code to the DNA of the present invention and which codes for a polypeptide which is at least part of the *Heliothis* ecdysone receptor transactivation domain, DNA binding domain, hinge domain, ligand binding domain and/or carboxy terminal region.

The DNA of the present invention may be cDNA or DNA which is in an isolated form.

According to another aspect of the present invention there is provided a polypeptide comprising the *Heliothis* ecdysone receptor or a fragment thereof, wherein said polypeptide is substantially free from other proteins with which it is ordinarily associated, and which is coded for by any of the DNA of the present invention.

According to another aspect of the present invention there is provided a polypeptide which has the amino acid sequence of Seq ID No. 4 or any allelic variant or derivative thereof, wherein Seq ID No. 4 gives the amino acid sequence of the HEcR polypeptide.

According to another aspect of the present invention there is provided a polypeptide which has part of the amino acid sequence of Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the HEcR ligand binding domain.

According to another aspect of the present invention there is provided a polypeptide
5 which has part of the amino acid sequence of Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the HEcR DNA binding domain.

According to yet another aspect of the present invention there is provided a polypeptide which has part of the amino acid sequence of Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the HEcR transactivation domain.

10 According to a further aspect of the present invention there is provided a polypeptide which has the amino acid sequence of a part of Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the HEcR hinge domain.

According to a still further aspect of the present invention there is provided a polypeptide which has the amino acid sequence of a part of Seq ID No. 4 or any allelic variant
15 or derivative thereof, which sequence provides the HEcR carboxy terminal region.

For the avoidance of doubt, spliced variants of the amino acid sequences of the present invention are included in the present invention.

Preferably, said derivative is a homologous variant which has conservative amino acid changes. By conservation amino acid changes we mean replacing an amino acid from one of
20 the amino acid groups, namely hydrophobic, polar, acidic or basic, with an amino acid from within the same group. An examples of such a change is the replacement of valine by methionine and vice versa.

According to another aspect of the present invention there is provided a fusion polypeptide comprising at least one of the polypeptides of the present invention functionally
25 linked to an appropriate non-*Heliothis* ecdysone receptor domain(s).

According to an especially preferred embodiment of the present invention the HEcR ligand binding domain of the present invention is fused to a DNA binding domain and a transactivation domain.

According to another embodiment of the present invention the DNA binding domain is
30 fused to a ligand binding domain and a transactivation domain.

According to yet another embodiment of the present invention the transactivation domain is fused to a ligand binding domain and a DNA binding domain.

The present invention also provides recombinant DNA encoding for these fused polypeptides.

35 According to an especially preferred embodiment of the present invention there is provided recombinant nucleic acid comprising a DNA sequence encoding the HEcR ligand

binding domain functionally linked to DNA encoding the DNA binding domain and transactivation domain from a glucocorticoid receptor.

According to yet another aspect of the present invention there is provided recombinant nucleic acid comprising a DNA sequence comprising a reporter gene operably
5 linked to a promoter sequence and a hormone response element which hormone response element is responsive to the DNA bonding domain encoded by the DNA of the present invention.

According to another aspect of the present invention there is provided a construct transformed with nucleic acid, recombinant DNA, a polypeptide or a fusion polypeptide of the
10 present invention. Such constructs include plasmids and phages suitable for transforming a cell of interest. Such constructs will be well known to those skilled in the art.

According to another aspect of the present invention there is provided a cell transformed with nucleic acid, recombinant DNA, a polypeptide, or a fusion polypeptide of the present invention.

15 Preferably the cell is a plant, fungus or mammalian cell.

For the avoidance of doubt fungus includes yeast.

The present invention therefore provides a gene switch which is operably linked to a foreign gene or a series of foreign genes whereby expression of said foreign gene or said series of foreign genes may be controlled by application of an effective exogenous inducer.

20 Analogs of ecdysone, such as Muristerone A, are found in plants and disrupt the development of insects. It is therefore proposed that the receptor of the present invention can be used in plants transformed therewith as an insect control mechanism. The production of the insect-damaging product being controlled by an exogenous inducer. The insect-damaging product can be ecdysone or another suitable protein.

25 The first non-steroidal ecdysteroid agonists, dibenzoyl hydrazines, typified by RH-5849 [1,2-dibenzoyl, 1-tert-butyl hydrazide], which is commercially available as an insecticide from Rohm and Haas, were described back in 1988. Another commercially available compound in this series is RH-5992 [tebufenozide, 3,5-dimethylbenzoic acid 1-1 (1,1-dimethylethyl)-2(4-ethylbenzoyl) hydrazide]. These compounds mimic
30 20-hydroxyecdysone (20E) in both *Manduca sexta* and *Drosophila melanogaster*. These compounds have the advantage that they have the potential to control insects using ecdysteroid agonists which are non-steroidal. Further Examples of such dibenzoyl hydrazines are given in US Patent No. 5,117,057 to Rohm and Haas, and Oikawa et al, Pestic Sci, 41, 139-148 (1994). However, it will be appreciated that any inducer of the gene switch of the
35 present invention, whether steroidal or non-steroidal, and which is currently or becomes available, may be used.

The gene switch of the present invention, then, when linked to an exogenous or foreign gene and introduced into a plant by transformation, provides a means for the external regulation of expression of that foreign gene. The method employed for transformation of the plant cells is not especially germane to this invention and any method suitable for the target
5 plant may be employed. Transgenic plants are obtained by regeneration from the transformed cells. Numerous transformation procedures are known from the literature such as agroinfection using *Agrobacterium tumefaciens* or its Ti plasmid, electroporation, microinjection or plants cells and protoplasts, microprojectile transformation, to mention but a few. Reference may be made to the literature for full details of the known methods.

10 Neither is the plant species into which the chemically inducible sequence is inserted particularly germane to the invention. Dicotyledonous and monocotyledonous plants can be transformed. This invention may be applied to any plant for which transformation techniques are, or become, available. The present invention can therefore be used to control gene expression in a variety of genetically modified plants, including field crops such as canola,
15 sunflower, tobacco, sugarbeet, and cotton; cereals such as wheat, barley, rice, maize, and sorghum; fruit such as tomatoes, mangoes, peaches, apples, pears, strawberries, bananas and melons; and vegetables such as carrot, lettuce, cabbage and onion. The switch is also suitable for use in a variety of tissues, including roots, leaves, stems and reproductive tissues.

In a particularly preferred embodiment of the present invention, the gene switch of the
20 present invention is used to control expression of genes which confer resistance herbicide resistance and/or insect tolerance to plants.

Recent advances in plant biotechnology have resulted in the generation of transgenic plants resistant to herbicide application, and transgenic plants resistant to insects. Herbicide tolerance has been achieved using a range of different transgenic strategies. One well
25 documented example in the herbicide field is the use the bacterial xenobiotic detoxifying gene phosphinothricin acetyl transferase (PAT) from *Streptomyces hydroscopicus*. Mutated genes of plant origin, for example the altered target site gene encoding acetolactate synthase (ALS) from *Arabidopsis*, have been successfully utilised to generate transgenic plants resistant to herbicide application. The PAT and ALS genes have been expressed under the control of
30 strong constitutive promoter. In the field of insecticides, the most common example to-date is the use of the Bt gene.

We propose a system where genes conferring herbicide and/or insect tolerance would be expressed in an inducible manner dependent upon application of a specific activating chemical. This approach has a number of benefits for the farmer, including the following:

- 35 1. Inducible control of herbicide and/or insect tolerance would alleviate any risk of yield penalties associated with high levels of constitutive expression of herbicide and/or insect resistance genes. This may be a particular problem as early stages of growth

where high levels of transgene product may directly interfere with normal development. Alternatively high levels of expression of herbicide and/or insect resistance genes may cause a metabolic drain for plant resources.

2. The expression of herbicide resistance genes in an inducible manner allows the herbicide in question to be used to control volunteers if the activating chemical is omitted during treatment.
3. The use of an inducible promoter to drive herbicide and/or insect resistance genes will reduce the risk of resistance becoming a major problem. If resistance genes were passed onto weed species from related crops, control could still be achieved with the herbicide in the absence of inducing chemical. This would particularly be relevant if the tolerance gene conferred resistance to a total vegetative control herbicide which would be used (with no inducing chemical) prior to sowing the crop and potentially after the crop has been harvested. For example, it can be envisaged that herbicide resistance cereals, such as wheat, might outcross into the weed wild oats, thus conferring herbicide resistance to this already troublesome weed. A further example is that the inducible expression of herbicide resistance in sugar beet will reduce the risk of wild sugar beet becoming a problem. Similarly, in the field of insect control, insect resistance may well become a problem if the tolerance gene is constitutively expressed. The use of an inducible promoter will allow a greater range of insect resistance control mechanisms to be employed.

This strategy of inducible expression of herbicide resistance can be achieved with a pre-spray of chemical activator or in the case of slow acting herbicides, for example N-phosphonomethyl-glycine (commonly known as glyphosate), the chemical inducer can be added as a tank mix simultaneously with the herbicide. Similar strategies can be employed for insect control.

This strategy can be adopted for any resistance conferring gene/corresponding herbicide combination, which is, or becomes, available. For example, the gene switch of the present invention can be used with:

1. Maize glutathione S-transferase (GST-27) gene (see our International Patent Publication No WO90/08826), which confers resistance to chloroacetanilide herbicides such as acetochlor, metolachlor and alachlor.
2. Phosphinotricin acetyl transferase (PAT), which confers resistance to the herbicide commonly known as glufosinate.
3. Acetolactate synthase gene mutants from maize (see our International Patent Publication No WO90/14000) and other genes, which confer resistance to sulphonyl urea and imidazolinones.

4. Genes which confer resistance to glyphosate. Such genes include the glyphosate oxidoreductase gene (GOX) (see International Patent Publication No. WO92/00377); genes which encode for 5-enolpyruvyl-3-phosphoshikimic acid synthase (EPSPS), including Class I and Class II EPSPS, genes which encode for mutant EPSPS, and
5 genes which encode for EPSPS fusion peptides such as that comprised of a chloroplast transit peptide and EPSPS (see for example EP 218 571, EP 293 358, WO91/04323, WO92/04449 and WO92/06201); and genes which are involved in the expression of CPLyase.

Similarly, the strategy of inducible expression of insect resistance can be adopted for
10 any tolerance conferring gene which is, or becomes, available.

The gene switch of the present invention can also be used to controlled expression of foreign proteins in yeast and mammalian cells. Many heterologous proteins for many applications are produced by expression in genetically engineered bacteria, yeast cells and other eucaryotic cells such as mammalian cells.

15 As well as the obvious advantage in providing control over the expression of foreign genes in such cells, the switch of the present invention provides a further advantage in yeasts and mammalian cells where accumulation of large quantities of an heterologous protein can damage the cells, or where the heterologous protein is damaging such that expression for short periods of time is required in order to maintain the viability of the cells.

20 Such an inducible system also has applicability in gene therapy allowing the timing of expression of the therapeutic gene to be controlled. The present invention is therefore not only applicable to transformed mammalian cells but also to mammals *per se*.

A further advantage of the inducible system of the present invention in mammalian cells is that, because it is derived from an insect, there is less chance of it being effected by
25 inducers which effect the natural mammalian steroid receptors.

In another aspect of the present invention the gene switch is used to switch on genes which produce potentially damaging or lethal proteins. Such a system can be employed in the treatment of cancer in which cells are transformed with genes which express proteins which are lethal to the cancer. The timing of the action of such proteins on the cancer cells can be
30 controlled using the switch of the present invention.

The gene switch of the present invention can also be used to switch genes off as well as on. This is useful in disease models. In such a model the cell is allowed to grow before a specific gene(s) is switched off using the present invention. Such a model facilitates the study of the effect of a specific gene(s).

35 Again the method for producing such transgenic cells is not particularly germane to the present invention and any method suitable for the target cell may be used; such methods are known in the art, including cell specific transformation.

As previously mentioned, modulation of gene expression in the system appears in response to the binding of the HEcR to a specific control, or regulatory, DNA element. A schematic representation of the HEcR gene switch is shown in Figure 6. For ease of reference, the schematic representation only shows three main domains of the HEcR, namely the transactivation domain, DNA binding domain and the ligand binding domain. Binding of a ligand to the ligand binding domain enables the DNA binding domain to bind to the HRE resulting in expression (or indeed repression) of a target gene.

The gene switch of the present invention can therefore be seen as having two components. The first component comprising the HEcR and a second component comprising an appropriate HRE and the target gene. In practice, the switch may conveniently take the form of one or two sequences of DNA. At least part of the one sequence, or one sequence of the pair, encoding the HEcR protein. Alternatively, the nucleic acid encoding the HEcR can be replaced by the protein/ polypeptide itself.

Not only does the switch of the present invention have two components, but also one or more of the domains of the receptor can be varied producing a chimeric gene switch. The switch of the present invention is very flexible and different combinations can be used in order to vary the result/to optimise the system. The only requirement in such chimeric systems is that the DNA binding domain should bind to the hormone-response element in order to produce the desired effect.

The glucocorticoid steroid receptor is well characterised and has been found to work well in plants. A further advantage of this receptor is that it functions as a homodimer. This means that there is no need to express a second protein such as the ultraspiracle in order to produce a functional receptor. The problem with the glucocorticoid steroid receptor is that ligands used to activate it are not compatible with agronomic practice.

In a preferred aspect of the present invention the receptor comprises glucocorticoid receptor DNA binding and transactivation domains with a *Heliothis* ligand binding domain according to the present invention. The response unit preferably comprising the glucocorticoid hormone response element and the desired effect gene. In the Examples, for convenience, this effect gene took the form of a reporter gene. However, in non-test or non-screen situations the gene will be the gene which produces the desired effect, for example produces the desired protein. This protein may be a natural or exogenous protein. It will be appreciated that this chimeric switch combines the best features of the glucocorticoid system, whilst overcoming the disadvantage of only being inducible by a steroid.

In another preferred embodiment, the *Heliothis* ligand binding domain is changed, and preferably replaced with a non-*Heliothis* ecdysone receptor ligand binding domain. For example, we have isolated suitable sequences from *Spodoptera exigua*.

Thus, according to another aspect of the present invention there is provided DNA having the sequence shown in Seq ID No. 6.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 6, which encodes for the *Spodoptera* ecdysone
5 ligand binding domain.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 6, which encodes for the *Spodoptera* ecdysone hinge domain.

The present invention also provides the polypeptides coded for by the above DNA
10 sequences of Seq ID No. 6.

A further advantage with such chimeric systems is that they allow you to choose the promoter which is used to drive the effector gene according to the desired end result. For example, placing the foreign gene under the control of a cell specific promoter can be particularly advantageous in circumstances where you wish to control not only the timing of
15 expression, but also which cells expression occurs in. Such a double control can be particularly important in the areas of gene therapy and the use of cytotoxic proteins.

Changing the promoter also enables gene expression to be up- or down-regulated as desired.

Any convenient promoter can be used in the present invention, and many are known in
20 the art.

Any convenient transactivation domain may also be used. The transactivation domain VP16 is a strong activator from Genentech Inc., and is commonly used when expressing glucocorticoid receptor in plants. Other transactivation domains derived for example from plants or yeast may be employed.

25 In a preferred embodiment of the present invention, the DNA binding domain is the glucocorticoid DNA binding domain. This domain is commonly a human glucocorticoid receptor DNA binding domain. However, the domain can be obtained from any other convenient source, for example, rats.

According to another aspect of the present invention there is provided a method of
30 selecting compounds capable of being bound to an insect steroid receptor superfamily member comprising screening compounds for binding to a polypeptide or fusion polypeptide of the present invention, and selecting said compounds exhibiting said binding.

According to another aspect of the present invention there is provided a compound selected using the method of the present invention.

35 According to another aspect of the present invention there is provided an agricultural or pharmaceutical composition comprising the compound of the present invention.

According to yet another aspect of the present invention there is provided the use of the compound of the present invention as a pesticide, pharmaceutical and/or inducer of the switch. It will be appreciated that such inducers may well be useful as insecticides in themselves.

5 According to a further aspect of the present invention there is provided a method of producing a protein or peptide or polypeptide comprising introducing into a cell of the present invention, a compound which binds to the ligand binding domain in said cell.

Various preferred features and embodiments of the present invention will now be described by way of non-limiting example with reference to the accompanying examples and
10 figures, in which figures:

Figure 1 (Sequence ID No. 1) shows the DNA sequence amplified from first strand cDNA made from mRNA isolated from *Heliothis virescens* Fourth instar larvae. The underlined sequences refer to the position of the degenerate oligonucleotides. At the 5' end the sequence matches that of the oligonucleotide while at the 3' end 12 nucleotides of the
15 original oligonucleotide are observed;

Figure 2 (Sequence ID No. 2) shows the DNA sequence contained within the clone pSK19R isolated from a random primed cDNA *Heliothis virescens* library; Sequence is flanked by EcoRI sites;

Figure 3 (Sequence ID No. 3) shows the DNA sequence contained within the clone
20 pSK16.1 isolated from a random primed cDNA *Heliothis virescens* library;

Figure 4 (Sequence ID No. 4) DNA sequence of 5'RACE products (in bold) fused to sequence of clone pSK16.1. The ORF (open reading frame) giving rise to the *Heliothis virescens* ecdysone receptor protein sequence is shown under the corresponding DNA sequence;

25 Figure 5 (Sequence ID No. 5) shows the protein sequence alignment of the ecdysone receptors DmEcR (*Drosophila melanogaster*), CtEcR (*Chironomus tentans*), BmEcR (*Bombyx mori*), MsEcR (*Manduca sexta*), AaEcR (*Aedes aegypti*) and HvEcR (*Heliothis virescens*). "*" indicates conserved amino acid residue. "." indicates a conservative amino acid exchange;

30 Figure 6 shows a model of an embodiment of the glucocorticoid/*Heliothis* ecdysone chimeric receptor useable as a gene switch;

Figure 7 shows a plasmid map of the clone pcDNA319R. The three other mammalian expression vectors were constructed in the same way and look similar but for the size of the insert;

35 Figure 8 shows a plasmid map of the reporter construct used to analyse the activity of the *Heliothis virescens* ecdysone receptor;

Figure 9 is a graph which shows the effect of Muristerone A and RH5992 in reporter activity in HEK293 cells co-transfected with pcDNA3H3KHEcR alone (filled bars) or with α RXR (stripped bars);

Figure 10 shows a plasmid map of the Maize expression vector containing the
5 Glucocorticoid receptor (HG1 or pMF6HG1PAT);

Figure 11 shows a plasmid map of the maize expression vector containing the chimeric glucocorticoid/*Drosophila* ecdysone receptor pMF6GREcRS;

Figure 12 shows a plasmid map of the maize expression vector containing the chimeric glucocorticoid/*Heliothis* ecdysone receptor pMF6GRHEcR;

10 Figure 13 shows a plasmid map of the plant reporter Plasmid containing the glucocorticoid response elements fused to the -60 S35CaMV promoter fused to GUS, p221.9GRE6;

Figure 14 shows a plasmid map of the plant reporter plasmid containing the glucocorticoid response elements fused to the -46 S35CaMV promoter fused to GUS,
15 p221.10GRE6;

Figure 15 shows a graph showing the effect of Muristerone A and Dexamethasone in Maize AXB protoplasts transformed with pMF6HG1PAT (GR) and p221.9GRE6 (reporter);

Figure 16 shows a graph showing the effect of Muristerone A and Dexamethasone in Maize AXB protoplasts transformed with pMF6GREcRS (effector) and p221.9GRE6
20 (reporter);

Figure 17 shows a graph showing the effect of Muristerone A and Dexamethasone in Maize AXB protoplasts transformed with pMF6GRHEcR (effector) and p221.9GRE6 (reporter);

Figure 18 shows a graph showing the effect of RH5849 in Maize AXB protoplasts
25 transformed with pMF6GREcRS (effector) and p221.9GRE6 (reporter);

Figure 19 shows a graph showing the effect of RH5992 in Maize AXB protoplasts transformed with pMF6GREcRS (effector) and p221.9GRE6 (reporter);

Figure 20 shows a graph showing the effect of RH5992 in Maize AXB protoplasts transformed with pMF6GRHEcR (effector) and p221.9GRE6 (reporter);

30 Figure 21 shows a graph which shows the dose response effect of RH5992 in Maize AXB protoplasts transformed with pMF6GRHEcR (effector) and p221.9GRE6 (reporter);

Figure 22 shows a plasmid map of the tobacco expression vector containing the chimeric glucocorticoid/*Drosophila* ecdysone receptor, pMF7GREcRS;

Figure 23 shows a plasmid map of the tobacco expression vector containing the
35 chimeric glucocorticoid/*Heliothis* ecdysone receptor, pMF7GRHEcR;

Figure 24 shows a graph which shows the effect of RH5992 in Tobacco mesophyll protoplasts transformed with pMF6GRHEcR (Effector) and p221.9GRE6 (reporter);

Figure 25 shows a plasmid map of the mammalian expression vector containing the chimeric glucocorticoid/*Heliothis* ecdysone receptor, pcDNA3GRHEcR;

Figure 26 shows a plasmid map of the reporter plasmid pSWGRE4;

Figure 27 shows a graph which shows a RH5992 dose response curve of CHO cells
5 transfected with pcDNA3GRHEcR and pSWGRE4;

Figure 28 shows a graph which shows the effect of Muristerone A and RH5992 on
HEK293 cells co-transfected with pcDNA3GRHEcR and pSWGRE4;

Figure 29 shows a plasmid map of the binary vector ES1;

Figure 30 shows a plasmid map of the binary vector ES2;

10 Figure 31 shows a plasmid map of the binary vector ES3;

Figure 32 shows a plasmid map of the binary vector ES4;

Figure 33 shows a plasmid map of the effector construct TEV-B112 made to express
the HEcR ligand binding domain in yeast;

Figure 34 shows a plasmid map of the effector construct TEV8 made to express the
15 HEcR ligand binding domain in yeast;

Figure 35 shows a plasmid map of the effector construct TEVVP16-3 made to express
the HEcR ligand binding domain in yeast;

Figure 36 shows a plasmid map of the mammalian expression vector containing the
chimeric glucocorticoid VP16/*Heliothis* ecdysone receptor, pcDNA3GRVP16HEcR;

20 Figure 37 shows a plasmid map of the maize expression vector containing the chimeric
glucocorticoid VP16/*Heliothis* ecdysone receptor, pMF6GRVP16HEcR;

Figure 38 shows a plasmid map of the maize expression vector containing the chimeric
glucocorticoid VP16/*Heliothis* ecdysone receptor, pMF7GRVP16HEcR;

Figure 39 shows a graph which shows the effect of RH5992 in Maize AXB
25 protoplasts transformed with pMF6GRVP16HEcR (effector) and p221.9GRE6 (reporter);

Figure 40 (Sequence ID No. 6) shows the DNA sequence of the hinge and ligand
binding domains of the *Spodoptera exigua* ecdysone receptor;

Figure 41 (Sequence ID No. 7) shows the protein sequence alignment of the *Heliothis*
19R and *Spodoptera* SEcR *Taq* clone hinge and ligand binding domains. "*" indicates
30 conserved amino acid residue. "." indicates a conservative amino acid exchange;

Figure 42 shows a graph which shows the effect of RH5992 on Tobacco mesophyll
protoplasts transformed with pMF7GRHEcR (effector) and either p221.9GRE6 (Horizontal
strips) or p221.10GRE6 (vertical strips).

Example I - Cloning of the *Heliothis* Ecdysone Receptor**A. Probe generation**

- 5 The rational behind the generation of the probe to isolate *Heliothis* homologues to the steroid/thyroid receptor superfamily members was based on comparing the sequences of developmentally regulated steroid/thyroid receptor superfamily members. The sequences available showed a highly conserved motif within the DNA binding domain of the RAR and THR (thyroid) receptors. The motifs were used to design degenerate oligonucleotides for
- 10 PCR amplification of sequences derived from cDNA template produced from tissue expected to express developmentally regulated steroid/thyroid receptor superfamily members (ie. larval tissues).

The sense oligonucleotide is based on the peptide sequence CEGCKGFF which at the DNA level yields an oligonucleotide with degeneracy of 32 as shown below :

15

ZnFA5' 5' TGC GAG GGI TGC AAG GAI TTC TT 3'
 T A T A T

The antisense oligonucleotide is based on the reverse complement nucleotide sequence derived from the peptide:

20

CQECRLKK
 S R

for which four sets of degenerate oligos were made. Namely:

25

ZnFA3' 5' TTC TTI AGI CGG CAC TCT TGG CA 3'
 T A T C A

ZnFB3' 5' TTC TTI AAI CGG CAC TCT TGG CA 3'
 T A T C A

30

ZnFC3' 5' TTC TTI AGI CTG CAC TCT TGG CA 3'
 T A T C A

ZnFD3' 5' TTC TTI AAI CTG CAC TCT TGG CA 3'
 T A T C A

35

The PCR amplification was carried out using a randomly primed cDNA library made from mRNA isolated from 4th and 5th instar *Heliothis virescens* larvae. The amplification

was performed using 10^8 pfus (plaque forming units) in 50mM KCl, 20mM Tris HCl pH 8.4, 15mM MgCl₂, 200mM dNTPs (an equimolar mixture of dCTP, dATP, dGTP and dTTP), 100ng of ZnFA5' and ZnF3' mixture. The conditions used in the reaction followed the hot start protocol whereby the reaction mixture was heated to 94°C for 5 minutes after which 1 U of Taq polymerase was added and the reaction allowed to continue for 35 cycles of 93°C for 50 seconds, 40°C for 1 minute and 73°C for 1 minute 30 seconds. The PCR products were fractionated on a 2%(w/v) agarose gel and the fragment migrating between 100 and 200bp markers was isolated and subcloned into the vector pCRII (Invitrogen). The sequence of the insert was determined using Sequenase (USB).

The resulting sequence was translated and a database search carried out. The search recovered sequences matching to the DNA binding domain of the *Drosophila* ecdysone receptor, retinoic acid receptor and the thyroid receptor. Thus, the sequence of the insert in this plasmid, designated pCRIIZnf, is a *Heliothis* ecdysone cognate sequence (Figure 1) and was used to screen a cDNA library in order to isolate the complete open reading frame.

B. Library screening

The randomly primed cDNA 4th/5th Instar *Heliothis virescens* library was plated and replicate filter made from the plates. The number of plaques plated was 500,000. The insert fragment of pCRIIZnf was reamplified and 50ng were end labelled using T4 Polynucleotide Kinase (as described in Sambrook et al 1990).

The filter were prehybridised using 0.25%(w/v) Marvel, 5 X SSPE and 0.1%(w/v) SDS at 42°C for 4 hours. The solution in the filters was then replaced with fresh solution and the denatured probe added. The hybridisation was carried out overnight at 42°C after which the filter were washed in 6 X SSC + 0.1%(w/v) SDS at 42°C followed by another wash at 55°C. The filter were exposed to X-ray film (Kodak) for 48 hours before processing.

The developed film indicated the presence of one strong positive signal which was plaque purified and further characterised. The lambda ZAP II phage was in vivo excised (see Stratagene Manual) and the sequence determined of the resulting plasmid DNA. The clone known as pSK19R (or 19R) contained a 1.933kb cDNA fragment with an open reading frame of 467 amino acids (Figure 2). pSK19R was deposited with the NCIMB on 20 June 1995 and has been accorded the deposit No NCIMB 40743.

Further analysis of pSK19R revealed that a 340 bp EcoRI-fragment mapping at the 5' end of pSK19R has strong and significant similarities to a *Drosophila* cDNA encoding glyceraldehyde-3-phosphate dehydrogenase. In order to isolate the correct 5' end sequence belonging to *Heliothis*, the random primed library was re-screened using a probe containing the 5' end of the pSK19R belonging to *Heliothis* ecdysone receptor. The probe was made by PCR using the sense oligonucleotide HecRH3C (5' aattaagcttcaccatgccgttaccatgccaccgaca

3') and antisense oligonucleotide HecrNdeI (5' cttcaaccgacactcctgac 3'). The PCR was carried out as described by Hirst et al., 1992) where the amount of radioisotope used in the labelling was 50uCi of a ³²P-dCTP and the PCR was cycled for 1 minute at 94°C, 1 minute at 60°C and 1 minute at 72°C for 19 cycles. The resulting 353bp radio labelled DNA fragment was denatured and added to prehybridised filters as described for the isolation of pSK19R. The library filters were made from 15 plates each containing 50000 pfus. The library filters were hybridised at 65°C and washed in 3XSSPE + 0.1%SDS at 65°C twice for 30 minutes each. The filters were further washed with 1XSSPE + 0.1%SDS for 30 minutes and exposed to X-ray film (Kodak) overnight. The film was developed and 16 putative positive plaques were picked. The plaques were re-plated and hybridised under the exact same conditions as the primary screen resulting in only one strong positive. The strong positive was consistently recognised by the probe and was plaque purified and *in vivo* excised. The resulting plasmid pSK16.1 was sequenced (Seq 1D3) which revealed that the 5' end of the clone extended by 205 bp and at the 3' end by 653 bp and resulting in a DNA insert of 2.5 kb. Conceptual translation of the 205 bp yielded 73 amino acids with high similarity to the *Drosophila*, *Aedes aegypti*, *Manduca* and *Bombyx* sequences of the ecdysone receptor B1 isoform. However, the whole of the 5' end sequence is not complete since a Methionine start site was not found with a stop codon in frame 5' of the methionine. In order to isolate the remainder of the 5' end coding sequences a 5'RACE protocol (Rapid Amplification of cDNA Ends) was carried out using the BRL-GIBCO 5'RACE Kit. Two types of cDNA were synthesised where the first one used a specific oligonucleotide :
 16PCR2A 5' cagctccaggccgcatctcg3'
 and the second type used random hexamers (oligonucleotide containing 6 random nucleotides). Each cDNA was PCR amplified using the oligonucleotides anchor primer :
 BRL-GIBCO 5' cuacuacuagggccacgcgtcgactagtagcggiiggggiig 3'
 and 16PCR2A and cycled for 1 minute at 94°C, 1 minute at 60°C and 1 minute at 72°C for 35 cycles. The reaction conditions were 20mM Tris-HCl (pH8.4), 50mM KCl, 1.5mM MgCl₂, 400nM of each anchor and 16PCR2A primers, 200mM dNTPs (dATP,dCTP,dGTP and dTTP) and 0.02 U/ml *Taq* DNA polymerase. Dilutions of 1:50 of the first PCR reactions were made and 1ml was use in a second PCR with oligonucleotides UAP :
 (Universal Amplification Primer 5' caucaucaugggccacgcgtcgactagtag 3')
 and 16RACE2 :
 (5' acgtcacctcagacgagctctccattc 3').

The conditions and cycling were the same as those followed for the first PCR. Samples of each PCR were run and a Southern blot carried out which was probed with a 5' specific primer :
 (16PCR1 5' cgctgtataacaacggaccattc 3').

This primer is specific for the 5' most sequence of pSK16.1 and was hybridised at 55°C using the standard hybridisation buffer. The filter was washed at 55°C 3 times in 3XSSPE + 0.1%SDS and exposed to X-ray film for up to 6 hours. The developed film revealed bands recognised by the oligonucleotide migrating at 100bp and 500bp (relative to the markers). A sample of the PCR reaction (4 in total) was cloned into the pCRII vector in the TA cloning kit (Invitrogen). Analysis of 15 clones from 4 independent PCRs yielded sequence upstream of pSK16.1 (Figure 4).

Translation of the ORF results in a 575 amino acid protein with high similarity in the DNA and ligand binding domains when compared to the ecdysone receptor sequences of *Drosophila*, *Aedes aegypti*, *Chironomus tentans*, *Manduca sexta* and *Bombyx mori* (Figure 5). Interestingly, the N-terminal end of the *Heliothis* sequence has an in frame methionine start which is 20 amino acids longer than that reported for *Drosophila*, *Aedes aegypti* and *Manduca sexta*. However, the extended N-terminal end in the *Heliothis* EcR does not have similarity to that of *Bombyx mori*. Finally, the C-terminal end of the different B1 isoform ecdysone receptor sequences diverge and do not have significant similarity.

C. Northern Blot Analysis

The sequence identified by screening the library is expected to be expressed in tissues undergoing developmental changes, thus mRNA from different developmental stages of *H. virescens* were isolated and a northern blot produced. The mRNAs were isolated from eggs, 1st, 2nd, 3rd, 4th and 5th instar larvae, pupae and adults. The northern blot was hybridised with a NdeI/XhoI DNA fragment from pSK19R encompassing the 3' end of the DNA binding domain through to the end of the ligand binding domain. The hybridisation was carried out in 1%(w/v)Marvel, 5X SSPE, 0.1%(w/v) SDS at 65°C for 18 to 24 hours. The filters were washed in 3X SSPE + 0.1%(w/v) SDS and 1X SSPE + 0.1%(w/v) SDS at 65°C. The filter was blotted dry and exposed for one to seven days. The gene recognises two transcripts (6.0 and 6.5 kb) which appear to be expressed in all stages examined; however, the levels of expression differ in different stages. It should be noted that the same two transcripts are recognised by probes specific to the DNA binding domain and the ligand binding domain, indicating that the two transcripts arise from the same gene either by alternative splicing or alternative use of polyadenylation sites.

In summary, adult and 5th instar larvae have lower levels of expression while all other tissues have substantial levels of expression.

Example II Expression of *Heliothis* ecdysone receptor in Mammalian cells

To demonstrate that the cDNA encodes a functional ecdysone receptor, effector constructs were generated containing the HEcR under the control of the CMV (cytomegalovirus) promoter, and the DNA expressed in mammalian cells.

Effector constructs

A first mammalian expression plasmid was constructed by placing a HindIII/NotI pSK19R fragment into the pcDNA3 HindIII/NotI vector resulting in pcDNA3 19R (Figure 7).

A second effector plasmid was constructed wherein the non-coding region of the cDNA 19R was deleted and a consensus Kozak sequence introduced. The mutagenesis was carried out by PCR amplifying a DNA fragment with the oligo HecRH3C :

5'aattaagcttcaccatgccgttaccaatgccaccgaca 3'

containing a unique HindIII restriction enzyme recognition site followed by the mammalian Kozak consensus sequence, and HecRNdeI :

5'cttcaaccgacactcctgac 3'.

The resulting 353bp PCR fragment was restriction enzyme digested with HindIII and NdeI, gel purified and ligated with 19R NdeI/NotI fragment into a pcDNA3 HindIII/NotI vector resulting in pcDNA3HecR.

A third effector construct was made with the 5' end sequences of pSK16.1 by PCR. The PCR approach involved PCR amplifying the 5' end sequences using a 5' oligonucleotide containing a HindIII restriction cloning site, the Kozak consensus sequence followed by nucleotide sequence encoding for a Methionine start and two Arginines to be added to the 5' end of the amplified fragment :

(16H3K 5' attaatgcttgccgcatgcccgcgacgtgtataacaacggaccattc 3'),
the 3' oligonucleotide used was HecrNdeI. The resulting fragment was restriction enzyme digested, gel purified and subcloned with an NdeI/NotI 19R fragment into pcDNA3 NdeI/NotI vector. The plasmid was named pcDNA3H3KHecR.

A fourth effector construct was produced which contains the extended N-terminal end sequence obtained from the 5'RACE experiment. Thus, a PCR approach was followed to introduce the new 5' end sequences in addition to a consensus Kozak sequence and a HindIII unique cloning sequence. The sense oligonucleotide used was RACEH3K :

5' attaatgcttgccgcatgtccctcggcgctcgttgatac 3',

while the antisense primer was the same as that used before (HecrNdeI). The cloning strategy was the same as used for the pcDNA3H3KHecR to give rise to pcDNA3RACEH3KHecR.

The PCR mutagenesis reactions were carried out in the same manner for all constructs. The PCR conditions used were 1 minute at 94°C, 1 minute at 60°C and 1 minute

at 72°C for 15 cycles. The reactions conditions were 50mM Tris-HCl (pH8.4), 25mM KCl, 200mM dNTPs (dATP, dCTP, dGTP and dTTP), 200nM of each oligonucleotide and 2.5U/Reaction of *Taq* DNA polymerase. For each construct at least 5 independent PCR reactions were carried out and several clones were sequenced to insure that at least one is mutation free.

Reporter construct

The reporter plasmid to be co-transfected with the expression vector contained 4 copies of the Hsp27 ecdysone response element (Riddihough and Pelham, 1987) fused to B-globin promoter and the B-Galactosidase gene. The tandem repeats of the ecdysone response element were synthesised as two complementary oligonucleotides which when annealed produced a double stranded DNA molecule flanked by an *SpeI* site at the 5' end and a *ClaI* site at the 3' end :

Recr3A

5'ctagtagacaaggggttcaatgcacttgccaataagcttagacaaggggttcaatgcacttgccaatgaattcagacaaggggttcaatgcacttgccaatctgcagagacaaggggttcaatgcacttgccaatat 3'

Recr3B

5'cgatattggacaagtcattgaacccttgctctgcagattggacaagtcattgaacccttgctgaattcattggacaagtcattgaacccttgcttaagcttattggacaagtcattgaacccttgctta 3'.

The resulting 135bp DNA fragment was ligated to the vector pSWBGAL *SpeI/ClaI* resulting in pSWREcR4 (Figure 8). The co-transfection of the two plasmid should result in B-galactosidase activity in the presence of ligand. The experiment relies upon the presence of RXR (a homologue of ultraspiracle) in mammalian cells for the formation of an active ecdysone receptor.

Mammalian transfection methods

Transfections of mammalian cell lines (CHO-K1 Chinese hamster ovary)- ATCC number CCL61 or cos-1 (Monkey cell line) were performed using either calcium phosphate precipitation (Gorman, Chapter 6 of "DNA cloning: a practical approach. Vol 2 D.M. Glover ed/.(1985) IRL Press, Oxford) or using LipofectAMINE (Gibco BRL Cat. No. 18324-012, following manufacturers instructions). Human Epithelial Kidney-293 cells were transfected using analogous methods.

Results - Native HEcR drives transient reporter gene expression in mammalian cells

Co-transfection of pcDNA3H3KHEcR (Effector) and reporter constructs into Human Epithelial Kidney 293 cells (HEK293) in the presence of either Muristerone A or RH5992 resulted in a 2-3 fold induction of reporter activity compared to the no chemical controls (Figure 9). The HEK293 cells were used since they are known to have constitutive levels of α RXR which have been demonstrated to be necessary for *Drosophila* EcR activation by Muristerone A (Yao., *et al.*, 1993). Moreover, to further investigate the need for RXR

interactions, a α RXR was co-transfected into HEK293 cells (along with the effector and reporter) resulting in a 9 fold induction of reporter activity compared to the untreated cells (Figure 9). The co-transfection of α RXR with reporter and effector increased by four fold the reporter activity compared to cells transfected with effector and reporter alone. Induction was
5 observed both in the presence of either Muristerone A or RH5992. These data clearly demonstrate that the cDNA HEcR encodes a functional ecdysone receptor. Moreover, The ability of HEcR to complex with α RXR and bind Muristerone A or RH5992 provide evidence for the usage of the entire HEcR as a component of a mammalian gene switch. In particular, it offers the advantage of reducing uninduced expression of target gene since ecdysone
10 receptor and response elements are not present in mammalian cells.

Example III - Chimeric constructs and ligand validation in Maize Protoplasts

In order to apply the ecdysone receptor as an inducible system it was deemed necessary
15 to simplify the requirements of the system by avoiding the need of a heterodimer formation to obtain an active complex. The glucocorticoid receptor is known to form homodimers and chimeric constructs of the glucocorticoid receptor transactivating and DNA binding domains fused to the ecdysone receptor hinge and ligand binding domains have been shown to be active as homodimers in mammalian cells in the presence of Muristerone A (an ecdysone
20 agonist)(Christopherson et al., 1992). However, the chimeric receptor is not responsive to 20-hydroxyecdysone (Christopherson et al., 1992).

The analysis of the activation of the glucocorticoid/*Heliothis* ecdysone chimeric receptor entailed the production of two other control effector constructs. The first one of the constructs contained the intact glucocorticoid receptor while the second one contained a
25 glucocorticoid/*Drosophila* ecdysone chimeric receptor.

Effector constructs

(i) Glucocorticoid receptor Maize expression construct

The glucocorticoid receptor DNA for the Maize transient expression construct was produced via the polymerase chain reaction (PCR) of Human Fibrosarcoma cDNA (HT1080
30 cell line, ATCC#CC1121) library (Clontech)(see Hollenberg *et al.*, 1985). The PCR approach taken was to amplify the 2.7kb fragment encoding the glucocorticoid receptor in two segments. The first segment entails the N-terminal end up to and including the DNA binding domain while the second fragment begins with the hinge region (amino acid 500) thought to the end of the reading frame. Thus, the PCR primer for the N-terminal end segment was
35 designed to contain an EcoRI site and the Kozak consensus sequence for translation initiation

:
GREcoRI 5'attgaattccaccatggactccaaagaatcattaactc 3'.

The 3' end primer contains a XhoI site in frame with the reading frame at amino acid 500 of the published sequence :

GRXhoI 5' gagactcctgtagtggcctcgagcattcctttatttttc 3'.

The second fragment of the glucocorticoid receptor was produced with a 5' end oligonucleotide containing an XhoI site in frame with the open reading frame at the beginning of the hinge region (amino acid 500) :

GRHinge 5' attctcgagattcagcaggccactacaggag 3'

while the 3' end oligonucleotide contained an EcoRI site 400 bp after the stop codon :

GRStop 5' attgaattcaatgctatcgttaactatacaggg 3'.

The glucocorticoid receptor PCR was carried out using Vent polymerase (Biolabs) under hot start conditions followed by 15 cycles of denaturing (94°C for 1 minute), annealing (66°C for 1 minute) and DNA synthesis (72°C for 3 minute). The template was produced by making first strand cDNA as described in the TA cloning kit (Invitrogen) after which the PCR was carried out in 10mM KCl, 10mM (NH₄)₂SO₄, 20mM TRIS-HCl pH 8.8, 2 mM MgSO₄, 0.1% (v/v) Triton X-100, 200 mM dNTPs, 100ng of each Primer and 2 U of Vent Polymerase. The PCR products was restriction enzyme digested with EcoRI and XhoI and subcloned into pBluescript SK (pSK) EcoRI. The resulting plasmid pSKHGI was sequenced and found to lack any mutations from the published sequences (apart from those introduced in the PCR primers) (Hollenberg et al., 1985).

The 2.7kb EcoRI fragment was subcloned into the vector pMF6PAT EcoRI resulting in pMF6HGIPAT (Figure 10).

(ii) Maize expression construct containing a Glucocorticoid/ *Drosophila* ecdysone chimeric receptor.

The glucocorticoid receptor portion of the chimeric receptor was isolated from pSKHGI by producing a 1.5kb BamHI/XhoI restriction fragment containing the N-terminal end up to and including the DNA binding domain.

The *Drosophila* ecdysone receptor portion was isolated through PCR of first stand cDNA prepared from *Drosophila* adult mRNA. The PCR was carried out using a 5' oligonucleotide containing a SalI site (ie. *Drosophila* ecdysone receptor contains a XhoI site at the end of the ligand binding domain) which starts at the beginning of the hinge region : amino acid 330, Ecr8 attgtcgacaacggccggaatggctcgtcccgag 3'.

The 3' end oligonucleotide contains an BamHI site adjacent to the stop codon : Ecrstop 5' tcgggctttgtaggatacctaagccgtggtcgaatgctccgacttaac 3'.

The PCR was carried out under the conditions described for the amplification of the Glucocorticoid receptor and yielded a 1.6 kb fragment. The fragment was introduced into

pSK SalI/BamHI and the sequence determined and compared to the published one (Koelle et al., 1991).

The maize transient expression plasmid was produced by introducing into pMF6 BamHI vector the 1.5kb BamHI/XhoI glucocorticoid receptor fragment and the 1.6kb SalI/BamHI *Drosophila* receptor portion to yield the chimeric plasmid pMF6GREcRS (Figure 9).

(iii) Construction of the Glucocorticoid/*Heliothis* ecdysone chimeric receptor Maize transient expression plasmid.

The Glucocorticoid receptor portion of the chimera was produced as describe in Example II(ii). The production of the *Heliothis* ecdysone receptor portion involves the introduction of a SalI recognition site at the DNA binding/hinge domain junction (amino acid 229). The addition of the SalI site :

Hecrsal 5'attgtcgacaaaggcccgagtgcgtggtgccggag 3'

was achieved via PCR mutagenesis making use of an unique AccI site 107bp downstream of the junction point (or 1007 bp relative to Seq 1D No 4):

Hecracc 5' tcacattgcatgatgggaggcatg 3'.

The PCR was carried out using *Taq* polymerase (2.5 U) in a reaction buffer containing 100ng of template DNA (pSK19R), 100ng of Hecrsal and Hecracc, 20 mM TRIS-HCl pH 8.4, 50mM KCl, 10mM MgCl₂, 200mM dNTPs. The reaction was carried out with an initial denaturation of 3 minutes followed by 15 cycles of denaturation (1 minute at 94°C), annealing (1 minute at 60°C) and DNA synthesis (1 minute at 72°C). The DNA was restriction enzyme digested and subcloned into pSK SalI/SacI with the 1.2kb AccI/SacI 3' end HecR fragment to yield pSK HeCRDEF (or containing the hinge and ligand binding domains of the *Heliothis* ecdysone receptor). The construction of the maize transient expression plasmid containing the Glucocorticoid/*Heliothis* ecdysone chimeric receptor involved the ligation of pMF6 EcoRI/SacI with the 1.5kb EcoRI/XhoI fragment of Glucocorticoid receptor N-terminal end and the 1.2 kb SalI/SacI fragment of pSk HEcRDEF to yield pMF6GRHEcR (Figure 10).

Reporter plasmids

Two reporter plasmids were made by inserting the into p221.9 or p221.10 BamHI/HindIII vectors two pairs or oligonucleotides containing six copies of the glucocorticoid response element (GRE). The two sets of oligonucleotides were designed with restriction enzyme recognition sites so as to ensure insertion of the two pairs in the right orientation. The first oligonucleotide pair GRE1A/B is 82 nucleotides long and when annealed result in a DNA fragment flanked with a HindIII site at the 5' end and a SalI site at the 3' end : GRE1A

5'agcttcgactgtacaggatgttctagctactcgagtagctagaacatcctgtacagtcgagtagctagaacatcctgtacag 3'

GRE1B

5'cgactgtacaggatgttctagctactcgactgtacaggatgttctagctactcgagtcgctagaacatcctgta cagtcga 3'.

The second pair of oligonucleotides is flanked by a Sall site at the 5' end and a BamHI site at the the 3' end

5 GRE2A 5' tcgactagctagaacatcctgtacagtcgagtagctagaacatcctgt
acagtcgagtagctagaacatcctgtacag 3'

GRE2B 5'gacctgtacaggatgttctagctactcgactgtacaggatgttctagctactcgactgtacaggatgttctagctag 3'.

The resulting plasmids were named p221.9GRE6 (Figure 13) and p221.10GRE6 (Figure 14)(used in later Example). The difference between p221.9 and p221.10 plasmids is that p221.9 contains the -60 35SCaMV minimal promotor while p221.10 (p221.10GRE6) contains the -46 35SCaMV minimal promotor.

Method

Protoplasts were isolated from a maize suspension culture-derived from BE70 x A188 embryogenic callus material, which was maintained by subculturing twice weekly in MS0.5_{mod}. (MS medium supplemented with 3% sucrose, 690mg/l proline, 1g/l myo-inositol, 0.2g/l casein acid hydrolysate, 0.5mg/l 2,4-D, pH5.6). Cells from suspensions two days post subculture were digested in enzyme mixture (2.0% Cellulase RS, 0.2% Pectolyase Y23, 0.5M Mannitol, 5mM CaCl₂·2H₂O, 0.5% MES, pH5.6, ~660mmol/kg) using ~10ml/g cells, incubating at 25°C, dim light, rotating gently for ~2 hours. The digestion mixture was sieved sequentially through 250µm and 38µm sieves, and the filtrate centrifuged at 700rpm for 3.5 minutes, discarding the supernatant. The protoplasts were resuspended in wash buffer (0.358M KCl, 1.0mM NH₄NO₃, 5.0mM CaCl₂·2H₂O, 0.5mM KH₂PO₄, pH4.8, ~670mmol/kg) and pelleted as before. This washing step was repeated. The pellet was resuspended in wash buffer and the protoplasts were counted. Transformation was achieved using a Polyethylene glycol method based on Negrutiu et.al. Protoplasts were resuspended at 2 x 10⁶/ml in MaMg medium (0.4M Mannitol, 15mM MgCl₂, 0.1% MES, pH5.6, ~450mmol/kg) aliquotting 0.5ml / treatment (i.e. 1x10⁶ protoplasts / treatment). Samples were heat shocked at 45°C for 5 minutes then cooled to room temperature. 10µg each of p221.9GRE6 and pMF6HR1PAT (GR) (1mg/ml) / treatment were added and mixed in gently, followed by immediate addition of 0.5ml warm (~45°C) PEG solution (40% PEG 3,350MW in 0.4M Mannitol, 0.1M Ca(NO₃)₂, pH8.0), which was mixed in thoroughly but gently. Treatments were incubated at room temperature for 20-25 minutes, then 5ml 0.292M KCl (pH5.6, ~530mmol/kg) was added step-wise, 1ml at a time, with mixing. The treatments were incubated for a further 10-15 minutes prior to pelleting the protoplasts by centrifuging as before. Each protoplast treatment was resuspended in 1.5ml culture medium (MS medium, 2% sucrose, 2mg/l 2,4-D, 9% Mannitol, pH5.6, ~700mmol/kg) +/- 0.0001M dexamethasone (glucocorticoid). The samples were incubated in 3cm dishes at 25°C, dark, for 24-48 hours prior to harvesting. Fluorometric

assays for GUS activity were performed with the substrate 4-methylumbelliferyl-D-glucuronide using a Perkin-Elmer LS-35 fluorometer (Jefferson et al., 1987). Protein concentration of tissue homogenates were determined by the Bio-Rad protein assay (Bradford, 1976). The method was repeated for each effector construct.

5 Results

Reporter gene assay

A reporter gene construct (p221.9GRE6) was generated containing the GUS reporter gene under the control of a -60 CaMV 35S promoter with 6 copies of the glucocorticoid response element. To test this construct was functional in maize protoplasts a co-transformation assay was performed with the reporter construct p221.9GRE6 and the effector construct pMF6HR1PAT (GR) construct containing the entire glucocorticoid receptor.

Figure 15 shows that Reporter p221.9GRE6 alone or reporter plus effector pMF6HR1PAT (GR) with no activating chemical gave no significant expression. When reporter plus effector were co-transformed into maize protoplasts in the presence of 0.0001M dexamethasone (glucocorticoid), a significant elevation of marker gene activity was observed (Figure 15). The response is specific to glucocorticoid as the steroid Muristerone A does not lead to induced levels of expression. These studies clearly show the reporter gene construct p221.9GRE6 is capable of monitoring effector /ligand mediated gene expression.

15 Chimeric ecdysone effector constructs mediate inducible expression in maize transient protoplasts assays

A chimeric effector plasmid pMF6GREcRS was constructed, containing the ligand binding domain from the *Drosophila* ecdysone receptor and the DNA binding and transactivation domain from the glucocorticoid receptor. To confirm the reporter gene construct p221.9GRE6 could respond to a chimeric ecdysone effector construct, a series of co-transformation into maize protoplasts was performed.....

Figure 16 shows that reporter (p221.9GRE6) alone or reporter plus effector (pMF6GREcRS) with no activating chemical, gave no significant expression in maize protoplasts. When reporter plus effector were co-transformed into maize protoplasts in the presence of 100µM Muristerone A, a significant elevation of marker gene activity was observed. The response was specific to Muristerone A, as the steroid dexamethasone did not lead to induced levels of expression. These studies clearly showed the reporter gene construct p221.9GRE6 is capable of monitoring chimeric ecdysone effector /ligand mediated gene expression.

A second chimeric effector construct pMF6GRHEcR, was generated containing the ligand binding domain from *Heliothis* ecdysone receptor. When co-transformed into maize protoplasts with the reporter plasmid p221.9GRE6, no response to 100µM Muristerone or

100µM dexamethasone was observed (Figure 17). These data clearly show the *Drosophila* and *Heliothis* ligand binding domains exhibit different properties.

When the effector plasmid pMF6GREcRS, containing the ligand binding domain from *Drosophila*, was tested with the reporter p221.9GRE6 in presence of the non-steroidal ecdysone agonists RH5849 and RH5992 (mimic), no chemical induced reporter gene activity was observed (Figures 18 and 19).

When the effector plasmid pMF6GRHEcR, containing the ligand binding domain from *Heliothis*, was tested with the reporter p221.9GRE6 in presence of the non-steroidal ecdysone agonists RH5992 (mimic), significant chemical induced reporter gene activity was observed (Figure 20). These data demonstrate the ligand binding domain from *Heliothis* has different properties to the *Drosophila* receptor in that the former responded to the non-steroidal ecdysteroid agonist RH5992. Figure 21 demonstrates the effector plasmid pMF6GRHEcR confers RH5992 dependant inducibility on the reporter p221.9GRE6 in a dose responsive manner. Induction was observed in a range from 1µM-100µM RH5992.

Example IV - Testing of effector vectors in Tobacco protoplasts

The experiments carried out in the previous example demonstrated the specific effect of RH5992 (mimic) on pMF6GRHEcR in maize protoplasts. It is the aim in this example to show the generic application to plants of the glucocorticoid/*Heliothis* ecdysone chimeric receptor switch system. Tobacco shoot cultures cv. Samsun, were maintained on solidified MS medium + 3% sucrose in a controlled environment room (16 hour day / 8 hour night at 25°C, 55% R.H), were used as the source material for protoplasts. Leaves were sliced parallel to the mid-rib, discarding any large veins and the slices were placed in CPW13M 13% Mannitol, pH5.6, ~860mmol/kg) for ~1 hour to pre-plasmolyse the cells. This solution was replaced with enzyme mixture (0.2% Cellulase R10, 0.05% Macerozyme R10 in CPW9M (CPW13M but 9% Mannitol), pH5.6, ~600mmol/kg) and incubated in the dark at 25°C overnight (~16 hours). Following digestion, the tissue was teased apart with forceps and any large undigested pieces were discarded. The enzyme mixture was passed through a 75µm sieve and the filtrate was centrifuged at 600rpm for 3.5 minutes, discarding the supernatant. The pellet was resuspended in 0.6M sucrose solution and centrifuged at 600rpm for 10 minutes. The floating layer of protoplasts was removed using a pasteur pipette and diluted with CPW9M (pH5.6, ~560mmol/kg). The protoplasts were again pelleted by centrifuging at 600rpm for 3.5 minutes, resuspended in CPW9M and counted. A modified version of the PEG-mediated transformation above was carried out. Protoplasts were resuspended at 2x10⁶/ml in MaMg medium and aliquotted using 200µl / treatment (i.e. 4x10⁵ protoplasts / treatment). 20µg each of pMF6GRHEcRS and p221.9GRE6 DNA (1mg/ml) were added

followed by 200 μ l PEG solution and the solutions gently mixed. The protoplasts were left to incubate at room temperature for 10 minutes before addition of 5ml MSP19M medium (MS medium, 3% sucrose, 9% Mannitol, 2mg/l NAA, 0.5mg/l BAP, pH5.6, ~700mmol/kg) +/- 10 μ M RH5992. Following gentle mixing, the protoplasts were cultured in their tubes, lying horizontally at 25°C, light. The protoplasts were harvested for the GUS assay after ~24 hours.

Effector construct

(i) Construction of a Dicotyledonous expression vector

The vector produced is a derivative of pMF6. pMF6GREcRS was restriction enzyme digested with PstI to produce 3 fragments namely, 3.4(Adh Intronless pMF6), 3.2(GREcRS) and 0.5(Adh intron I) kb). Isolation and religation of the 3.4 and 3.2 kb fragments resulted in pMF7GREcRS (Figure 22). pMF7GREcRS was restriction enzyme digested with EcoRI/SacI resulting in the 3.4kb pMF7 EcoRI/SacI vector which when isolated and purified was ligated to a 1.5 kb EcoRI/XhoI N-terminal end of the glucocorticoid receptor and the 1.2 kb SalI/SacI *Heliothis* ecdysone C-terminal end sequences to produce pMF7GRHEcR (Figure 23).

Reporter plasmid

The reporter plasmids constructed for the maize transient experiments were the same as those used without alteration in the tobacco leaf protoplast transient expression experiments.

Results - Chimeric ecdysone effector constructs mediate inducible expression in tobacco transient protoplast assays

Experiments were performed to demonstrate that the effector plasmid pMF6GRHEcR can confer chemical dependant inducible expression on the reporter p221.9GRE6 in tobacco mesophyll protoplasts.

Figure 24 shows that reporter (p221.9GRE6) alone or reporter plus effector (pMF7GRHEcR) with no activating chemical, gave no significant expression in tobacco protoplasts. When reporter plus effector were co-transformed into tobacco protoplasts in the presence of 10 μ M RH5992, a significant elevation of marker gene activity was observed. These data show a chimeric ecdysone effector construct, containing the *Heliothis* ligand binding domain can confer non-steroidal ecdysteroid dependant expression on reporter gene constructs in both monocotyledonous and dicotyledonous species.

Example V - Chimeric activity in Mammalian cells

Effector constructs

- 5 (i) Construction of Glucocorticoid/*Heliothis* ecdysone chimeric receptor.

The mammalian expression vector used in this experiment was pcDNA3 (Invitrogen). The GRHEcR 2.7kb BamHI DNA fragment (isolated from pMF6GRHEcR) was introduced into the pcDNA3 BamHI vector. The recombinants were oriented by restriction enzyme mapping. The DNA sequence of the junctions was determined to ensure correct orientation and insertion (pcDNA3GRHEcR, Figure 25).

Reporter construct

The reporter plasmid for mammalian cell system was produced by taking pSWBGAL plasmid and replacing the CRESW SpeI/ClaI fragment for a synthetic 105 bp DNA fragment containing 4 copies of the glucocorticoid response element (GRE) and flanked by SpeI at the 15 5' end and AflII at the 3' end.

The oligonucleotides were synthesised using the sequences :

GREspeI

5'ctagttgtacaggatgttctagctactcgagtagctagaacatcctgtacagtcgagtagctagaacatcctgtacagtcgagtagct
agaacatcctgtacac 3'

20 GREafl2

5'ttaagtgtacaggatgttctagctactcgactgtacaggatgttctagctactcgactgtacaggatgttctagctactcgagtagcta
gaacatcctgtacaa 3'.

The two oligonucleotides were purified annealed and ligated to pSWBGAL SpeI/AflII to produce pSWGREG4 (Figure 26).

25 Results - Chimeric HEcR drives transient reporter gene expression in mammalian cells

No expression was detected when a reporter gene construct pSWGREG4, comprising of a minimal β -globin promoter containing four copies of the glucocorticoid response element, fused to a β -galactosidase reporter gene, was introduced into CHO cells. Similarly, no expression was detected when pSWGREG4 and an effector plasmid pCDNA3GRHEcR, 30 containing the transactivation and DNA binding domain from the glucocorticoid receptor and the ligand binding domain from the *Heliothis* ecdysone receptor, under the control of the CMV promoter were co-transformed into CHO-K1 or HEK293 cells. When co-transformed CHO (Figure 27) and HEK293 cells (Figure 28) were incubated in the presence of the non-steroidal ecdysone agonists RH5992 (mimic), significant chemical induced reporter gene 35 activity was observed. Equally, induction of reporter activity was observed when HEK293 cells transfected with pcDNA3GRHEcR and reporter were treated with Muristerone A (Figure 28).

Example VI - Screening system allows new chemical activators and modified ligand binding domains to be tested in Mammalian cells

5 The basis of a screening system are in place after the demonstration that the chimeric receptor was activated in the presence of RH5992. A screen was carried out using CHO cells transiently transfected with both pSWGREG4 (reporter) and pcDNA3GRHEcR (effector) constructs. In the first instance 20 derivatives compounds of RH5992 were screened. It was
10 observed that 7 out of the 20 compounds gave an increased reporter gene activity compared to untreated cells. A second screen was carried out in which 1000 randomly selected compounds were applied to transiently transfected CHO cells. Two compounds were found to activate reporter gene activity above that from the untreated controls. The second screen
15 suggest that this cell based assay is a robust and rapid way to screen a small library of compounds, where a thousand compounds can be put through per week.

Example V - Stably transformed Tobacco plants

Stable Tobacco vectors

20 The components of the stable Tobacco vectors were put together in pBluescript prior to transfer into the binary vector. The production of stable transformed plants entails the production of a vector in which both components of the switch system (ie. effector and reporter) are placed in the same construct to then introduce into plants.

 The methodology described below was used to produce four different stable Tobacco vectors. The method involves three steps:

25

1. pBluescript SK HindIII/EcoRI vector was ligated to either GRE6-4635SCaMVGUSNOS HindIII/EcoRI (from p221.10GRE6) or GRE6-6035SCaMVGUSNOS HindIII/EcoRI (from p221.9GRE6) resulting in plasmid pSK-46 and pSK-60.

30

2. This step involves the addition of the chimeric receptor (35SGRHEcRNOS or 35SGRVP16HEcRNOS) to pSK-60 or pSK-46. Thus a pSK-60 (or pSK-46) XbaI vector was ligated with either the 3.4kb 35SGRHEcRNOS XbaI or the 3.0kb 35SGRVP16HEcRNOS XbaI DNA fragment to produce pSKES1 (pSKGRE6-6035SCaMVGUSNOS-35SGRHEcRNOS), pSKES2 (pSKGRE6-4635SCaMVGUSNOS-35SGRHEcRNOS), pSKES3 (pSKGRE6-6035SCaMVGUSNOS-35SGRVP16HEcRNOS) and pSKES4 (pSKGRE6-4635SCaMVGUSNOS-35SGRVP16HEcRNOS).

35

3. Transfer from pBluescript based vectors to binary vectors. The transfer of the ES1 (Figure 29) ES2 (Figure 30), ES3 (Figure 31) or ES4 (Figure 32) DNA fragments into the binary vector JR1 involves five steps:

5

(i) Restriction enzyme digestion of pSKES1 (ES2, ES3, and ES4) with ApaI and NotI to liberate the insert from the vector pBluescript.

(ii) The two DNA fragments were BamHI methylated for 2 hours at 37°C in TRIS-HCl, MgCl₂, 80uM SAM (S-adenosylmethionine) and 20 U of BamHI methylase.

10 (iii) Ligate a ApaI/NotI linker onto the fragment. The linker was designed to have an internal BamHI site :

ApaBNot1 5' cattggatccttagc 3' and

ApaBNot2 5'ggccgctaaggatccaatgggcc 3'.

(iv) Restriction enzyme digest the protected and linkered fragment with BamHI and
15 fractionate the products on a 1%(w/v) Agarose gel. The protected DNA fragment (5.5kb) was cut out of the gel and purified.

(v) A ligation of JRI BamHI vector with the protected band was carried out to produce JRIES1 (JRIES2, JRIES3 or JRIES4). The DNA of the recombinant was characterised by restriction mapping and the sequence of the junctions determined.

20 The plant transformation construct pES1, containing a chimeric ecdysone receptor and a reporter gene cassette, was transferred into Agrobacterium tumefaciens LBA4404 using the freeze/thaw method described by Holsters et al. (1978). Tobacco (Nicotiana tabacum cv Samsun) transformants were produced by the leaf disc method (Bevan, 1984). Shoots were regenerated on medium containing 100mg/l kanamycin. After rooting, plantlets were
25 transferred to the glasshouse and grown under 16 hour light/ 8 hour dark conditions.

Results - Chimeric ecdysone effector constructs mediate inducible expression in stably tobacco plants

Transgenic tobacco plants were treated in cell culture by adding 100µM RH5992 to MS media. In addition seedlings were grown hydroponically in the presence or absence of
30 RH5992. In further experiments 5mM RH5992 was applied in a foliar application to 8 week old glasshouse grown tobacco plants. In the three methods described uninduced levels of GUS activity were comparable to a wild type control, while RH5992 levels were significantly elevated.

Ecdysone switch modulation and optimisation**Example VIII - Yeast indicator strains for primary screen of chemical libraries**

5

A set of yeast indicator strains was produced to use as a primary screen to find chemicals which may be used in the gene switch. The properties of the desired chemicals should include high affinity resulting in high activation but with different physico-chemical characteristics so as to increase the scope of application of the technology. Moreover, the production of this strain also demonstrates the generic features of this switch system.

10

Effector vector

A base vector for yeast YCp15Gal-TEV-112 was generated containing:

Backbone - a modified version of pRS315 (Sikorski and Hieter (1989) Genetics 122, 19-27)- a shuttle vector with the LEU2 selectable marker for use in yeast;

15

ADH1 promoter (BamHI- Hind III fragment) and ADH1 terminator (Not I- Bam HI fragment) from pADNS (Colicelli et al PNAS 86, 3599-3603);

DNA binding domain of GAL4 (amino acids 1-147; GAL4 sequence is Laughon and Gesteland 1984) Mol. Cell Biol. 4, 260-267) from pSG424 (Sadowski and Ptashne (1989) Nuc. Acids Res. 17, 7539);

20

Activation domain - an acidic activation region corresponding to amino acids 1-107 of activation region B112 obtained from plasmid pB112 (Ruden et al (1991) Nature 350, 250-252).

The plasmid contains unique Eco RI, Nco I and Xba I sites between the DNA binding domain and activation domains.

25

Into this vector a PCR DNA fragment of the *Heliothis* ecdysone receptor containing the hinge, ligand binding domains and the C-terminal end was inserted. The 5' oligonucleotide is flanked by an NcoI restriction recognition site and begins at amino acid 259 :

HecrNcoI 5' aattccatggtacgacgacagtagacgatcac 3'.

The 3' oligonucleotide is flanked by an XbaI site and encodes for up to amino acid 571:

30

HecRXbaI 5' ctgaggtctagagacggtggcggcgccgccc 3'.

The PCR was carried out using vent polymerase with the conditions described in Example IA. The fragment was restriction enzyme digested with NcoI and XbaI purified and ligated into YCp15GALTEV112 NcoI/XbaI vector to produce YGALHeCRB112 or TEV-B112 (Figure 34). In order to reduce constitutive activity of the YGALHeCRB112 plasmid a YGALHeCR plasmid was produced in which the B112 activator was deleted by restriction enzyme digesting YGALHeCRB112 with XbaI/SpeI followed by ligation of the resulting

35

vector (ie. SpeI and XbaI sites when digested produce compatible ends)(TEV-8, Figure 33). An effector plasmid was constructed whereby the B112 transactivating domain was excised from YGalHecRB112 with XbaI and replaced with the VP16 transactivation domain DNA fragment (encoding amino acids 411 and 490 including the stop codon). The resulting vector was named YGalHecRVP16 or TEVVP16-3 (Figure 35).

Reporter construction for yeast

The *S. cerevisiae* strain GGY1::171 (Gill and Ptashne (1987) Cell 51, 121-126), YT6::171 (Himmelfarb et al (1990) Cell 63, 1299-1309) both contain reporter plasmids consisting of the GAL4-responsive GAL1 promoter driving the *E. coli* B-galactosidase gene. These plasmids are integrated at the URA3 locus. The reporter strain YT6::185 contains the reporter plasmid pJP185 (two synthetic GAL4 sites driving the B-galactosidase gene) integrated at the URA3 locus of YT6 (Himmelfarb et al). (Note- the parental strains YT6 and GGY1 have mutations in the GAL4 and GAL80 genes, so the reporter genes are inactive in the absence of any plasmids expressing GAL4 fusions).

Yeast assay

Standard transformation protocols (Lithium acetate procedure) and selection of colonies by growth of cells on selective media (leucine minus medium in the case of the YCp15Gal-TEV-112 plasmid)- as described in Guthrie and Fink (1991) Guide to Yeast Genetics and Molecular Biology: Methods in Enzymology Vol. 194 Academic Press) and the reporter gene assay is a modification of that described in Ausabel et al (1993) Current Protocols in Molecular Biology (Wiley) Chapter 13).

Results - Automated screening system allows new chemical activators and modified ligand binding domains to be tested in yeast

An effector vector pYGALHEcRB112 has been generated containing a GAL4 DNA binding domain, a B112 activation domain and the ligand binding region from *Heliothis virescens*. In combination with a GAL reporter vector, pYGALHEcRB112 form the basis of a rapid, high throughput assay which is cheap to run. This cell-based assay in yeast (*Saccharomyces cerevisiae*) will be used to screen for novel non-steroidal ecdysone agonists which may of commercial interest as novel insecticides or potent activators of the ecdysone gene switch system. The demonstration of an efficient system to control gene expression in a chemical dependant manner, forms the basis of an inducible system for peptide production in yeast.

The yeast screening system forms the basis of a screen for enhanced ligand binding using the lac Z reporter gene vector to quantitatively assay the contribution of mutation in the ligand binding domain. Alternatively, enhanced ligand binding capabilities or with a selection cassette where the lac Z reporter is replaced with a selectable marker such as uracil (URA 3), tryptophan (Trp1) or leucine (Leu2), and histidine (His). Constructs based on

pYGALHEcRB112 with alterations in the ligand binding domain are grown under selection conditions which impair growth of yeast containing the wild type ligand binding domain. Those surviving in the presence of inducer are retested and then sequenced to identify the mutation conferring resistance.

5

Example IX - Optimisation of chimeric receptor using a strong transactivator

Construction of mammalian expression plasmid with chimeric receptor containing Herpes Simplex VP16 protein sequences.

10 The construction of this chimeric receptor is based on replacing the sequences encoding for the glucocorticoid receptor transactivating domain with those belonging to the VP16 protein of Herpes simplex. Thus PCR was used to generate three fragments all to be assembled to produce the chimeric receptor. The PCRs were carried out as described in Example II, iii. The first fragment includes the Kozak sequences and methionine start site of
15 the glucocorticoid receptor to amino acid 152 of the glucocorticoid receptor. The oligonucleotides used for the generation of this fragment included an EcoRI site at the 5' end:

GR1A 5' atatgaattccaccatggactccaaagaatc 3'

and at the 3' end a NheI restriction enzyme recognition site :

GR1B 5' atatgctagctgtgggggcagcagacacagcagtgg 3'.

20 The second fragment also belongs to the glucocorticoid receptor and begins with a NheI site in frame with amino acid 406 :

GR2A 5'atagctagctccagctcctcaacagcaacaac 3'

and ends with a XhoI site at amino acid 500 :

GR2B 5'atatctcgagcaattcctttatttttttc 3'.

25 The two fragments were introduced into pSKEcoRI/SacI in a ligation containing GR1A/B EcoRI/NheI, GR2A/B NheI/XhoI and HEcR SalI/SacI (from pSKHEcRDEF) to yield pSKGRDHEcR. The GR sequences and junctions of the ligation were found to be mutation free.

30 The third fragment to be amplified was a sequence between amino acid 411 to 490 of the herpes simplex VP16 protein. The amplified fragment was flanked with SpeI recognition sites. SpeI produces compatible ends to those of NheI sites. The oligonucleotides used :

VP16C 5' attactagtctgcggccccccgaccgat 3' and

VP16E 5' aattactagtcccaccgtactcgtcaattcc 3'

35 produced a 180 bp fragment which was restriction enzyme digested with SpeI and introduced into pSKGRAHEcR NheI vector to produce pSKGRVP16HEcR. The DNA from the latter was sequenced and found to be mutation free, the junctions were also shown to be in frame with those of the glucocorticoid receptor.

The 2.2 kb EcoRV/NotI GRVP16HEcR fragment was introduced into a pcDNA3 EcoRV/NotI vector resulting in pcDNA3GRVP16HEcR (Figure 36).

Construction of plant transient expression effector plasmids containing the chimeric receptor with VP16 sequences

- 5 The same procedure was carried out to clone the GRVP16HEcR DNA fragment into tobacco(pMF7b) and maize(pMF6) expression vectors. A 2.2kb BamHI DNA fragment was isolated from pcDNA3GRVP16HEcR and ligated in to the pMF6 BamHI (or pMF7b BamHI) vector to produce pMF6GRVP16HEcR (Figure 37) (or pMF7GRVP16HEcR) (Figure 38).

Results - Addition of strong activation domains enhance ecdysone switch system

- 10 The VP16 transactivation domain from herpes simplex virus has been added to a maize protoplast vector pMF6GRHEcR to generate the vector pMF6GRVP16HEcR. When co-transformed into maize protoplasts with the reporter construct p221.9GRE6, in the presence of 100µM RH5992, enhanced levels of expression were seen over pMF6GRHEcR. Figure 39, shows that RH5992 is able to induce GUS levels comparable to those observed
15 with the positive control (p35SCaMVGUS), moreover, a dose response effect is observable.

VP16 enhanced vectors (pES3 and pES4) have been generated for stable transformation of tobacco. Following transformation transgenic progeny containing pES3 and pES4, gave elevated GUS levels following treatment with RH5992, relative to comparable transgenic plants containing the non-VP16 enhanced vectors pES1 and pES2.

- 20 An enhanced mammalian vector pcDNA3GRVP16HEcR was prepared for transient transfection of mammalian cell lines. Elevated reporter gene activities were obtained relative to the effector construct (pcDNA3GRHEcR) without the VP16 addition.

- "Acidic" activation domains are apparently "universal" activators in eukaryotes (Ptashne (1988) Nature 335 683-689). Other suitable acidic activation domains which have
25 been used in fusions are the activator regions of GAL4 itself (region I and region II; Ma and Ptashne (Cell (1987) 48, 847-853), the yeast activator GCN4 (Hope and Struhl (1986) Cell 46, 885-894) and the herpes simplex virus VP16 protein (Triezenberg et al (1988) Genes Dev. 2, 718-729 and 730-742).

- Other acidic and non-acidic transcriptional enhancer sequences for example from plant
30 fungal and mammalian species can be added to the chimeric ecdysone receptor to enhance induced levels of gene expression.

Chimeric or synthetic activation domains can be generated to enhance induced levels of gene expression.

Example X - Optimisation by replacement of *Heliothis* ligand binding domain in chimeric effector for that of an ecdysone ligand binding domain of another species

5 Mutagenesis of the ecdysone ligand binding domain results in the increased sensitivity of the chimeric receptor for activating chemical. This can be achieved by deletions in the ligand binding domain, use of error prone PCR (Caldwell et al., PCR Meth. Applic 2, 28-33 1992), and in vitro DNA shuffling PCR (Stemmer, Nature 370, 389-391 1994). To enhance the efficacy of the listed techniques we have developed a screening system for enhanced levels
10 of induced expression (see below).

An alternative strategy to the mutation of a known ligand binding domain is to identify insect species which are particularly sensitive to ecdysteroid agonists. For example *Spodoptera exigua* is particularly sensitive to RH 5992. To investigate the role of the ecdysone receptor ligand binding domain in increased sensitivity to RH5992 we have isolated
15 corresponding DNA sequences from of *S. exigua* (Figure 40, Sequence ID No. 6). Figure 41, Sequence ID No. 7 shows a protein alignment of the hinge and ligand binding domains of the *Heliothis virescens* and *Spodoptera exigua* ecdysone receptors. The protein sequence between the two species is well conserved.

Results - Manipulation of the ligand binding domain leads to enhanced induced expression

20 Isolation of an ecdysone ligand binding domain from another lepidopteran species was carried out by using degenerate oligonucleotides and PCR of first strand cDNA (Perkin Elmer, cDNA synthesis Kit) of the chosen species. The degenerate oligonucleotides at the 5' end were HingxhoA and B and at the 3' end ligandxA/B

25 HingxhoA 5' attgctcgagaaagiccigagtgcgtigticc 3'

a t

HingxhoB 5' attgctcgagaacgiccigagtgtgtigticc 3'

a c

30 LigandxA 5' ttactcgagiactgccaiatctcttciaaggaa 3'

a t c a

ligandxB 5' ttactcgagiactgccaiatctcctciaaggaa 3'

a t t a

35

RNA was extracted from 4th instar larvae of *Spodoptera exigua* since *Spodoptera exigua* appears to be more sensitive to RH5992 than *Heliothis* (Smagghe and Degheele,

1994). The first strand cDNA was used in PCR reactions under the following conditions 20mM Tris-HCL (pH8.4), 50mM KCl, 1.5mM MgCl₂, 200mM dNTPs (dATP,dCTP,dGTP and dTTP) and 0.02 U/ml *Taq* DNA polymerase and in the presence of 1 µg of each Hinge (5' 3') and Ligand (5'3') oligonucleotides. The PCR cycling conditions were 94°C for 1 minute, 60°C for 2 minutes and 72°C for 1 minute and 35 cycles were carried out. A sample of the completed reaction was fractionated in a 1% agarose (w/v) 1 x TBE gel, and the resulting 900 bp fragment was subcloned into pCRII vector (Invitrogen). The resulting clone (pSKSEcR 1-10) were further characterised and sequenced.

10 Example X - Manipulation of reporter gene promoter regions can modulate chemical induced expression

The context of the effector response element in the reporter gene promoter can be used to modulate the basal and induced levels of gene expression. Six copies of the glucocorticoid response element were fused to 46 bp or 60 bp of the CaMV 35S promoter sequence. When used with the effector construct pMF7GRHEcRS the reporter gene construct containing 46 bp of the CaMV 35S promoter gave reduced basal and induced levels of GUS expression relative to the 60 bp reporter construct (Figure 42).

Constructs for plant transformation (pES1 and ES2) have been generated to demonstrate the size of minimal promoter can be used to modulate the basal and induced levels of gene expression in plants.

The number and spacing of response elements in the reporter gene promoter can be adjusted to enhance induced levels of trans-gene expression.

The utility of a two component system (effector and reporter gene cassettes) allows the spatial control of induced expression. Trans-gene expression can be regulated in an tissue specific, organ specific or developmentally controlled manner. This can be achieved by driving the effector construct from a spatially or temporally regulated promoter.

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- 35 and Rosenfeld, M.G. (1991) Cell 67, 1251-1266.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

(i) APPLICANT:

- (A) NAME: ZENECA LIMITED
(B) STREET: 15 STANHOPE GATE
(C) CITY: LONDON
(E) COUNTRY: UK
(F) POSTAL CODE (ZIP): W1Y 6LN

10

(ii) TITLE OF INVENTION: A GENE SWITCH

15

(iii) NUMBER OF SEQUENCES: 7

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

20

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: GB 9510759.5
(B) FILING DATE: 26-MAY-1995

25

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: GB 9513882.3
(B) FILING DATE: 07-JUL-1995

30

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: GB 9517316.7
(B) FILING DATE: 24-AUG-1995

35

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: GB 9605656.9
(B) FILING DATE: 18-MAR-1996

40

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 116 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: cDNA to mRNA

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(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Heliothis virescens*

55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

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(A) LENGTH: 1934 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

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(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Heliothis virescens*

10

(vii) IMMEDIATE SOURCE:

(B) CLONE: pSK19R

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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30	CCTGCAGCAG	ACACAACCCC	TACCTTCCAT	GCCGTTACCA	ATGCCACCGA	420
	ATCAGAAAAC	GAGTCAATGT	CATCAGGTCG	TGAGGAACTG	TCTCCAGCTT	480
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	GTTGAAGAAA	TGTCTTGCGG	TGGGCATGAG	GCCCAGAGTC	GTGGTGCCGG	780
	TGCAATGAAA	CGGAAAGAGA	AAAAGGCGCA	GAGGGAAAAA	GACAAATTGC	840
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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 2464 base pairs
 25 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: circular

 (ii) MOLECULE TYPE: cDNA
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 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: *Heliothis virescens*

 (vii) IMMEDIATE SOURCE:
 35 (B) CLONE: pSK16.1

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- (A) LENGTH: 2745 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 225..1955
 (D) OTHER INFORMATION: /codon_start= 225
 /product= "Heliothis eclydysone receptor"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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	TGCCTCCCAT CATGCAATGT GACCCTCCGC CCCCAGAGGC CGCTAGAATT CTGGAATGTG	1080
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	AGAACGTGCC CCCCCTCACT GCCAATCAGA AGTCGTTGAT CGCAAGGCTC GTGTGGTACC	1200
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	GTTATGTTTA TTTACCAAAA TTAACAATAA TACGTTGATT AACCTTTCGA GTATAATATT	2460
	GTGATGAGTC GTCCGCTGTC CACGTCGCCG TCACATGTTT GTTCTGATG CACACGTGAG	2520
40	GNGCGTTATC GTGTTTCATG GTTCCATCGT CCTGTGCCCC CGACCCTCGA CTAAATGAGT	2580
	AATTTAATTT ATTGCTGTGA TTACATTTTA ATGTGTTGAT TATCTACCAT AGGGTGATAT	2640
45	AAGTGTGTCT TATTACAATA CAAAGTGTGT GTCGTCGATA GCTTCCACAC GAGCAAGCCT	2700
	TTTGTTTAAG TGATTTACTG ACATGGACAC TCGACCCGGA ACTTC	2745

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 575 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

- 46 -

Met Ser Leu Gly Ala Arg Gly Tyr Arg Arg Cys Asp Thr Leu Ala Asp
 1 5 10 15
 5 Met Arg Arg Arg Trp Tyr Asn Asn Gly Gly Phe Gln Thr Leu Arg Met
 20 25 30
 Leu Glu Glu Ser Ser Ser Glu Val Thr Ser Ser Ser Ala Leu Gly Leu
 35 40 45
 10 Pro Pro Ala Met Val Met Ser Pro Glu Ser Leu Ala Ser Pro Glu Ile
 50 55 60
 Gly Gly Leu Glu Leu Trp Gly Tyr Asp Asp Gly Ile Thr Tyr Ser Met
 65 70 75 80
 15 Ala Gln Ser Leu Gly Thr Cys Thr Met Glu Gln Gln Gln Pro Gln Pro
 85 90 95
 Gln Gln Gln Pro Gln Gln Thr Gln Pro Leu Pro Ser Met Pro Leu Pro
 100 105 110
 20 Met Pro Pro Thr Thr Pro Lys Ser Glu Asn Glu Ser Met Ser Ser Gly
 115 120 125
 25 Arg Glu Glu Leu Ser Pro Ala Ser Ser Val Asn Gly Cys Ser Thr Asp
 130 135 140
 Gly Glu Ala Arg Arg Gln Lys Lys Gly Pro Ala Pro Arg Gln Gln Glu
 145 150 155 160
 30 Glu Leu Cys Leu Val Cys Gly Asp Arg Ala Ser Gly Tyr His Tyr Asn
 165 170 175
 35 Ala Leu Thr Cys Glu Gly Cys Lys Gly Phe Phe Arg Arg Ser Val Thr
 180 185 190
 Lys Asn Ala Val Tyr Ile Cys Lys Phe Gly His Ala Cys Glu Met Asp
 195 200 205
 40 Ile Tyr Met Arg Arg Lys Cys Gln Glu Cys Arg Leu Lys Lys Cys Leu
 210 215 220
 Ala Val Gly Met Arg Pro Glu Cys Val Val Pro Glu Asn Gln Cys Ala
 225 230 235 240
 45 Met Lys Arg Lys Glu Lys Lys Ala Gln Arg Glu Lys Asp Lys Leu Pro
 245 250 255
 50 Val Ser Thr Thr Thr Val Asp Asp His Met Pro Pro Ile Met Gln Cys
 260 265 270
 Asp Pro Pro Pro Pro Glu Ala Ala Arg Ile Leu Glu Cys Val Gln His
 275 280 285
 55 Glu Val Val Pro Arg Phe Leu Asn Glu Lys Leu Met Glu Gln Asn Arg
 290 295 300
 Leu Lys Asn Val Pro Pro Leu Thr Ala Asn Gln Lys Ser Leu Ile Ala
 305 310 315 320
 60 Arg Leu Val Trp Tyr Gln Glu Gly Tyr Glu Gln Pro Ser Glu Glu Asp
 325 330 335

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Leu Lys Arg Val Thr Gln Ser Asp Glu Asp Asp Glu Asp Ser Asp Met
 340 345 350
 5 Pro Phe Arg Gln Ile Thr Glu Met Thr Ile Leu Thr Val Gln Leu Ile
 355 360 365
 Val Glu Phe Ala Lys Gly Leu Pro Gly Phe Ala Lys Ile Ser Gln Ser
 370 375 380
 10 Asp Gln Ile Thr Leu Leu Lys Ala Cys Ser Ser Glu Val Met Met Leu
 385 390 395 400
 Arg Val Ala Arg Arg Tyr Asp Ala Ala Thr Asp Ser Val Leu Phe Ala
 405 410 415
 15 Asn Asn Gln Ala Tyr Thr Arg Asp Asn Tyr Arg Lys Ala Gly Met Ala
 420 425 430
 Tyr Val Ile Glu Asp Leu Leu His Phe Cys Arg Cys Met Tyr Ser Met
 435 440 445
 20 Met Met Asp Asn Val His Tyr Ala Leu Leu Thr Ala Ile Val Ile Phe
 450 455 460
 25 Ser Asp Arg Pro Gly Leu Glu Gln Pro Leu Leu Val Glu Asp Ile Gln
 465 470 475 480
 Arg Tyr Tyr Leu Asn Thr Leu Arg Val Tyr Ile Leu Asn Gln Asn Ser
 485 490 495
 30 Ala Ser Pro Arg Gly Ala Val Ile Phe Gly Glu Ile Leu Gly Ile Leu
 500 505 510
 Thr Glu Ile Arg Thr Leu Gly Met Gln Asn Ser Asn Met Cys Ile Ser
 515 520 525
 35 Leu Lys Leu Lys Lys Arg Lys Leu Pro Pro Phe Leu Glu Glu Ile Trp
 530 535 540
 40 Asp Val Ala Asp Val Ala Thr Thr Ala Thr Pro Val Ala Ala Glu Ala
 545 550 555 560
 Pro Ala Pro Leu Ala Pro Ala Pro Pro Ala Arg Pro Ala Thr Val
 565 570 575
 45

(2) INFORMATION FOR SEQ ID NO: 6:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 948 base pairs
 50 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: cDNA
 55 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: *Spodoptera exigua*
 60 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

AGGCCCGAGT GCGTGGTGCC AGAAAACCAG TGTGCAATGA AAAGGAAAGA GAAAAAGGCA

60

CAAAGGGAAA AAGACAAGTT GCCAGTCAGT ACAACGACAG TGGATGATCA CATGCCTCCC 120
ATTATGCAGT GTGATCCACC GCCTCCAGAG GCCGCAAGAA TTCACGAGGT GGTGCCACGA 180
5 TTCCTGAATG AAAAGCTAAT GGACAGGACA AGGCTCAAGA ATGTGCCCCC TCACTGCCAA 240
CCAGAAGTCC TTAATAGCGA GGCTGGTCTG GTACCAAGAA GGCTATGAAC AGCCATCAGA 300
10 AGAGGATCTA AAAAGAGTCA CACAGTCGGA TGAAGACGAA GAAGAGTCGG ACATGCCGTT 360
CCGTCAGATC ACCGAGATGA CGATCCTCAC AGTGCAGCTC ATTGTTGAAT TCGCTAAGGG 420
CCTACCAGCG TTCGCAAAGA TCTCACAGTC GGATCAGATC ACATTATTAA AGGCCTGTTC 480
15 GAGTGAGGTG ATGATGTTGC GAGTAGCTCG GCGGTACGAC GCGGCGACAG ACAGCGTGTT 540
GTTCCGCAAC AACCAGGCGT ACACCCGCGA CAACTACCGC AAGGCAGGCA TGGCCTACGT 600
20 CATCGAGGAC CTGCTGCACT TCTGCCGGTG CATGTACTCC ATGATGATGG ATAACGTCCA 660
CTATGCACTG CTCACTGCCA TCGTCATTTT CTCAGACCGA CCCGGGCTTG AGCTAACCTT 720
GTTGGTGGAG GAGATCCAGA GATATTACCT GAACACGCTG CGGGTGTACA TCCTGAACCA 780
25 GAACAGTCGG TCGCCGTGCT GCCCTGTCTAT CTACGCTAAG ATCCTCGGCA TCCTGACGGA 840
GCTGCGGACC CTGGGCATGC AGAACTCCAA CATGTGCATC TCACTCAAGC TGAAGAACAG 900
30 GAACGTGCCG CCGTTCTTCG AGGATATCTG GGACGTCTC GAGTAAAA 948

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
35 (A) LENGTH: 319 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: protein

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Arg Pro Glu Cys Val Val Pro Glu Asn Gln Cys Ala Met Lys Arg Lys
1 5 10 15
50 Glu Lys Lys Ala Gln Arg Glu Lys Asp Lys Leu Pro Val Ser Thr Thr
20 25 30
Thr Val Asp Asp His Met Pro Pro Ile Met Gln Cys Asp Pro Pro Pro
35 40 45
55 Pro Glu Ala Ala Arg Ile Leu Glu Cys Val Gln His Glu Val Val Pro
50 55 60
60 Arg Phe Leu Asn Glu Lys Leu Met Glu Gln Asn Arg Leu Lys Asn Val
65 70 75 80
Pro Pro Leu Thr Ala Asn Gln Lys Ser Leu Ile Ala Arg Leu Val Trp
85 90 95

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	Tyr	Gln	Glu	Gly	Tyr	Glu	Gln	Pro	Ser	Glu	Glu	Asp	Leu	Lys	Arg	Val	
				100					105					110			
5	Thr	Gln	Ser	Asp	Glu	Asp	Asp	Glu	Asp	Ser	Asp	Met	Pro	Phe	Arg	Gln	
			115					120					125				
	Ile	Thr	Glu	Met	Thr	Ile	Leu	Thr	Val	Gln	Leu	Ile	Val	Glu	Phe	Ala	
			130				135					140					
10	Lys	Gly	Leu	Pro	Gly	Phe	Ala	Lys	Ile	Ser	Gln	Ser	Asp	Gln	Ile	Thr	
	145					150					155					160	
	Leu	Leu	Lys	Ala	Cys	Ser	Ser	Glu	Val	Met	Met	Leu	Arg	Val	Ala	Arg	
15					165					170					175		
	Arg	Tyr	Asp	Ala	Ala	Thr	Asp	Ser	Val	Leu	Phe	Ala	Asn	Asn	Gln	Ala	
				180					185					190			
20	Tyr	Thr	Arg	Asp	Asn	Tyr	Arg	Lys	Ala	Gly	Met	Ala	Tyr	Val	Ile	Glu	
			195					200					205				
	Asp	Leu	Leu	His	Phe	Cys	Arg	Cys	Met	Tyr	Ser	Met	Met	Met	Asp	Asn	
25			210				215					220					
	Val	His	Tyr	Ala	Leu	Leu	Thr	Ala	Ile	Val	Ile	Phe	Ser	Asp	Arg	Pro	
	225					230					235					240	
	Gly	Leu	Glu	Gln	Pro	Leu	Leu	Val	Glu	Glu	Ile	Gln	Arg	Tyr	Tyr	Leu	
30					245					250					255		
	Asn	Thr	Leu	Arg	Val	Tyr	Ile	Leu	Asn	Gln	Asn	Ser	Ala	Ser	Pro	Arg	
				260					265					270			
35	Gly	Ala	Val	Ile	Phe	Gly	Glu	Ile	Leu	Gly	Ile	Leu	Thr	Glu	Ile	Arg	
			275					280					285				
	Thr	Leu	Gly	Met	Gln	Asn	Ser	Asn	Met	Cys	Ile	Ser	Leu	Lys	Leu	Lys	
40			290				295					300					
	Lys	Arg	Lys	Leu	Pro	Pro	Phe	Leu	Glu	Glu	Ile	Asp	Trp	Asp	Val		
	305					310						315					

CLAIMS

1. DNA comprising the sequence shown in Seq ID No. 2.
- 5 2. DNA comprising the sequence shown in Seq ID No. 3.
3. DNA comprising the sequence shown in Seq ID No. 4.
4. DNA comprising a sequence which shows 60% or more homology with the sequence
10 shown in Seq ID No 1, 2 or 3.
5. DNA according to claim 4 wherein said homology is in the range of 65% to 99%.
6. DNA which hybridises to the sequence shown in Seq. ID No. 2, 3 or 4, and which
15 codes for at least part of the *Heliothis* ecdysone receptor.....
7. DNA which is degenerate as a result of the genetic code to the DNA of any one of
claims 1 to 6 and which codes for a polypeptide which is at least part of the *Heliothis*
ecdysone receptor.
20
8. DNA comprising part of the sequence shown in Seq ID No. 2, and which codes for at
least part of the *Heliothis* ecdysone receptor ligand binding domain.
9. DNA comprising part of the sequence shown in Seq ID No. 3, and which codes for at
25 least part of the *Heliothis* ecdysone receptor ligand binding domain.
10. DNA comprising part of the sequence shown in Seq ID No. 4, and which codes for at
least part of the *Heliothis* ecdysone receptor ligand binding domain.
- 30 11. DNA comprising a sequence which shows 60% or more homology with the sequence
of claim 8, 9 or 10.
12. DNA according to claim 11 wherein said homology is in the range of 65% to 99%.
- 35 13. DNA which hybridises to the DNA of any one of claims 8 to 12 and which codes for
at least part of the *Heliothis* ecdysone receptor ligand binding domain.

14. DNA which is degenerate as a result of the genetic code to the DNA of any one of claims 8 to 12 and which codes for a polypeptide which is at least part of the *Heliothis* ecdysone receptor ligand binding domain.
- 5 15. DNA comprising part of the sequence shown in Seq ID No. 2, and which codes for at least part of the *Heliothis* ecdysone receptor DNA binding domain.
16. DNA comprising part of the sequence shown in Seq ID No. 3, and which codes for at least part of the *Heliothis* ecdysone receptor DNA binding domain.
- 10 17. DNA comprising part of the sequence shown in Seq ID No. 4, and which codes for at least part of the *Heliothis* ecdysone receptor DNA binding domain.
18. DNA comprising a sequence which shows 60% or more homology with the sequence of claim 15, 16 or 17.
- 15 19. DNA according to claim 18 wherein said homology is in the range of 65% to 99%.
20. DNA which hybridises to the DNA of any one of claims 15 to 19 and which codes for at least part of the *Heliothis* ecdysone receptor DNA binding domain.
- 20 21. DNA which is degenerate as a result of the genetic code to the DNA of any one of claims 15 to 19 and which codes for a polypeptide which is at least part of the *Heliothis* ecdysone receptor DNA binding domain.
- 25 22. DNA comprising part of the sequence shown in Seq ID No. 2, and which codes for at least part of the *Heliothis* ecdysone receptor transactivation domain.
23. DNA comprising part of the sequence shown in Seq ID No. 3, and which codes for at least part of the *Heliothis* ecdysone receptor transactivation domain.
- 30 24. DNA comprising part of the sequence shown in Seq ID No. 4, and which codes for at least part of the *Heliothis* ecdysone receptor transactivation domain.
- 35 25. DNA comprising a sequence which shows 60% or more homology with the sequence of claim 22, 23 or 24.

26. DNA according to claim 25 wherein said homology is in the range of 65% to 99%.
27. DNA which hybridises to the DNA of any one of claims 22 to 26 and which codes for at least part of the *Heliothis* ecdysone receptor transactivation domain.
- 5 28. DNA which is degenerate as a result of the genetic code to the DNA of any one of claims 22 to 26 and which codes for a polypeptide which is at least part of the *Heliothis* ecdysone receptor transactivation domain.
- 10 29. DNA comprising part of the sequence shown in Seq ID No. 2, and which codes for at least part of the *Heliothis* ecdysone receptor hinge domain.
30. DNA comprising part of the sequence shown in Seq ID No. 3, and which codes for at least part of the *Heliothis* ecdysone receptor hinge domain.
- 15 31. DNA comprising part of the sequence shown in Seq ID No. 4, and which codes for at least part of the *Heliothis* ecdysone receptor hinge domain.
- 20 32. DNA comprising a sequence which shows 60% or more homology with the sequence of claim 29, 30 or 31.
33. DNA according to claim 32 wherein said homology is in the range of 65% to 99%.
- 25 34. DNA which hybridises to the DNA of any one of claims 29 to 33 and which codes for at least part of the *Heliothis* ecdysone receptor hinge domain.
- 35 35. DNA which is degenerate as a result of the genetic code of the DNA of any one of claims 29 to 33 and which codes for a polypeptide which is at least part of the *Heliothis* ecdysone receptor hinge domain.
- 30 36. DNA having part of the sequence shown in Seq ID No. 2, and which codes for at least part of the *Heliothis* ecdysone receptor carboxy terminal region.
- 35 37. DNA having part of the sequence shown in Seq ID No. 3, and which codes for at least part of the *Heliothis* ecdysone receptor carboxy terminal region.

38. DNA having part of the sequence shown in Seq ID No. 4, and which codes for at least part of the *Heliothis* ecdysone receptor carboxy terminal region.
39. DNA comprising a sequence which shows 60% or more homology with the sequence
5 of claim 36, 37 or 38.
40. DNA according to claim 39 wherein said homology is in the range of 65% to 99%.
41. DNA which hybridises to the DNA of any one of claims 36 to 40 and which codes for
10 at least part of the *Heliothis* ecdysone receptor carboxy terminal region.
42. DNA which is degenerate as a result of the genetic code of the DNA of any one of claims 36 to 40 and which codes for a polypeptide which is at least part of the
15 *Heliothis* ecdysone receptor carboxy terminal region.
43. A polypeptide comprising the *Heliothis* ecdysone receptor or a fragment thereof, wherein said polypeptide is substantially free from other proteins with which it is ordinarily associated, and which is coded for by the DNA of any preceding claim.
- 20 44. A polypeptide comprising the amino acid sequence shown in Seq ID No. 4 or any allelic variant or derivative thereof.
45. A polypeptide comprising part of the amino acid sequence shown in Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the *Heliothis*
25 ecdysone receptor ligand binding domain.
46. A polypeptide comprising part of the amino acid sequence shown in Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the *Heliothis*
30 ecdysone receptor DNA binding domain.
47. A polypeptide comprising part of the amino acid sequence shown in Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the *Heliothis*
ecdysone receptor transactivation domain.
- 35 48. A polypeptide comprising part of the amino acid sequence shown in Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the *Heliothis* ecdysone receptor hinge domain.

49. A polypeptide comprising part of the amino acid sequence shown in Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the *Heliothis* ecdysone receptor carboxy terminal region.
50. A polypeptide according to any one of claims 44 to 49 wherein said derivative is a homologous variant which includes conservative amino acid changes.
51. DNA comprising the sequence shown in Seq ID No. 6.
52. DNA comprising a sequence which shows 60% or more homology with the sequence shown in Seq ID No. 6.
53. DNA according to claim 52 wherein said homology is in the range of 65% to 99%.
54. DNA which hybridises to the DNA sequence shown in Seq ID No. 6 and which codes for at least part of *Spodoptera* ecdysone receptor.
55. DNA which is degenerate as a result of the genetic code to the DNA of any one of claims 51 to 54.
56. DNA comprising part of the sequence shown in Seq ID No. 6, and which codes for at least part of the *Spodoptera* ecdysone receptor ligand binding domain.
57. DNA comprising a sequence which shows 60% or more homology with the sequence of claim 56.
58. DNA according to claim 57 wherein said homology is in the range of 65% to 99%.
59. DNA which hybridises to the DNA of any one of claims 56 to 58 and which codes for at least part of the *Spodoptera* ecdysone receptor ligand binding domain.
60. DNA which is degenerate as a result of the genetic code to the DNA of any one of claims 56 to 58 and which codes for at least part of the *Spodoptera* ecdysone receptor ligand binding domain.

61. DNA comprising part of the sequence shown in Seq ID No. 6, and which codes for at least part of the *Spodoptera* ecdysone receptor hinge domain.
- 5 62. DNA comprising a sequence which shows 60% or more homology with the sequence of claim 61.
63. DNA according to claim 62 wherein said homology is in the range of 65% to 99%.
- 10 64. DNA which hybridises to the DNA of any one of claims 61 to 63 and which codes for at least part of the *Spodoptera* ecdysone receptor hinge domain.
65. DNA which is degenerate as a result of the genetic code to the DNA of any one of claims 61 to 63 and which codes for at least part of the *Spodoptera* ecdysone receptor hinge domain.
- 15 66. A polypeptide coded for by the DNA of any one of claims 51 to 65.
67. A fusion polypeptide comprising the polypeptide of claim 45 or 50 (when dependent upon claim 45) and functionally linked to a DNA binding domain and a transactivation domain.
- 20 68. Recombinant DNA comprising the DNA of any one of claim 8 to 14 functionally linked to DNA encoding a DNA binding domain and a transactivation domain.
- 25 69. A fusion polypeptide according to claim 67 or recombinant DNA according to claim 68 wherein the DNA binding domain and/or transactivation domain is fungal, bacterial, plant or mammalian.
70. A fusion polypeptide or recombinant DNA according to claim 69 wherein the DNA binding domain is GAL4 or A1CR/A.
- 30 71. A fusion polypeptide or recombinant DNA according to claim 69 or 70 wherein the transactivation domain is VP16.
- 35 72. A fusion polypeptide or recombinant DNA according to claim 69 wherein the DNA binding domain and/or transactivation domain is from a steroid receptor superfamily member.

73. A fusion polypeptide or recombinant DNA according to claim 72 wherein the DNA binding domain and/or transactivation domain is from a glucocorticoid or a *Spodoptera* ecdysone receptor.
- 5
74. A recombinant DNA construct comprising recombinant DNA of any one of claims 68 to 73; and DNA which codes for a gene operably linked to a promoter sequence and a hormone response element, which is responsive to the DNA binding domain coded for by said recombinant DNA.
- 10
75. A fusion polypeptide comprising the polypeptide of claim 46 or 50 (when dependent upon claim 46) and functionally linked to a ligand binding domain and a transactivation domain.
- 15
76. Recombinant DNA comprising the DNA of any of claims 15 to 21 functionally linked to DNA encoding a ligand binding domain and a transactivation domain.
- 20
77. A fusion polypeptide according to claim 75 or recombinant DNA according to claim 76 wherein the ligand binding domain and/or transactivation domain is fungal, bacterial, plant or mammalian.
- 25
78. A fusion polypeptide or recombinant DNA according to claim 77 wherein the transactivation domain is VP16.
- 30
79. A fusion polypeptide or recombinant DNA according to claim 77 wherein the ligand binding domain and/or transactivation domain is from a steroid receptor superfamily member.
80. A fusion polypeptide or recombinant DNA according to claim 79 wherein the ligand binding domain and/or transactivation domain is from a glucocorticoid or *Spodoptera* ecdysone receptor.
- 35
81. A recombinant DNA construct comprising recombinant DNA of any one of claims 76 to 80; and DNA which codes for a gene operably linked to a promoter sequence and a hormone response element, which is responsive to the DNA binding domain coded for by said recombinant DNA.

82. A fusion polypeptide comprising the polypeptide of claim 47 or 50 (when dependent upon claim 47) and functionally linked to a ligand binding domain and a DNA binding domain.
- 5 83. Recombinant DNA comprising the DNA of any one of claims 22 to 28 functionally linked to DNA encoding a ligand binding domain and a DNA binding domain.
84. A fusion polypeptide according to claim 82 or recombinant DNA according to claim 10 83 wherein the ligand binding domain and/or DNA binding domain is fungal, bacterial, plant or mammalian.
85. A fusion polypeptide or recombinant DNA according to claim 84 wherein the DNA binding domain is GAL4 or A1CR/A.
- 15 86. A fusion polypeptide or recombinant DNA according to claim 84 wherein the ligand binding domain and/or DNA binding domain is from a steroid receptor superfamily member.
- 20 87. A fusion polypeptide or recombinant DNA according to claim 86 wherein the ligand binding domain and/or DNA binding domain is from a glucocorticoid or *Spodoptera* ecdysone receptor.
- 25 88. A recombinant DNA construct comprising recombinant DNA of any one of claims 82 to 87; and DNA which codes for a gene operably linked to a promoter sequence and a hormone response element, which is responsive to the DNA binding domain coded for by said recombinant DNA.
- 30 89. A recombinant DNA construct comprising DNA according to any one of claims 1 to 7; and DNA comprising a sequence which codes for a gene operably linked to a promoter sequence and at least one hormone response element which is responsive to the DNA binding domain coded for by said DNA of any one of claim 1 to 7.
- 35 90. A recombinant DNA construct according to any one of claims 74, 81, 88 and 89 wherein said promoter sequence codes for a constitutive, spatially or temporally regulating promoter.

91. A recombinant DNA construct according to any one of claims 74, 81, 88 and 89 wherein there is more than one copy of the hormone response element.
- 5 92. A cell transformed with the DNA of any one of claims 1 to 42, and 51 to 65; the polypeptide of any one of claims 43 to 50; the fusion polypeptide of any one of claims 67, 70 to 73, 75, 77 to 80, 82 and 84 to 87; the recombinant nucleic acid of any one of claims 68 to 73, 76 to 80 and 85 to 87; or the recombinant DNA construct of any one of claims 74, 81, 88 and 89.
- 10 93. A cell according to claim 92 wherein said cell is a plant, fungal or mammalian cell.
94. A plant, fungus or mammal comprising the recombinant DNA construct of any one of claims 74, 81, 88 and 89.
- 15 95. A method of selecting compounds capable of being bound to an insect steroid receptor superfamily member comprising screening compounds for binding to said polypeptide of any one of claims 43 to 50 or the fusion polypeptide of any one of claims 67, 70 to 73, 75, 77 to 80, 82 and 84 to 87, and selecting said compounds exhibiting said binding.
- 20 96. A compound selected using the method of claim 95.
97. An agricultural or pharmaceutical composition comprising the compound of claim 96.
- 25 98. Use of the compound of claim 96 as an agrochemical or a pharmaceutical.
99. A method of producing a protein, peptide or polypeptide comprising introducing into the cell of claim 92, a compound which binds to the ligand binding domain in said cell.

Fig.1.

Sequence ID 1

1 TGCG AGG GGT GCA AGG AGT TCT TCA GGC GGA GTG TAA CCA AAA ATG
 ACGC TCC CCA CGT TCC TCA AGA AGT CCG CCT CAC ATT GGT TTT TAC

46 CAG TGT ACA TAT GCA AAT TCG GCC ATG CTT GCG AAA TGG ATA TGT
 GTC ACA TGT ATA CGT TTA AGC CGG TAC GAA CGC TTT ACC TAT ACA

91 ATA TGC GGA GAA AAT GCC AAG AGT A
 TAT ACG CCT CTT TTA CGG TTC TCA T

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Fig.2.

Sequence ID 2

1	TCC	ACT	GGT	GTT	TTC	ACC	ACC	ACA	GAA	AAG	GCC	TCT	GCT	CAT	TTA
	AGG	TGA	CCA	CAA	AAG	TGG	TGG	TGT	CTT	TTC	CGG	AGA	CGA	GTA	AAT
46	GAG	GGT	GGT	GCT	AAG	AAG	GTC	ATC	ATC	TCC	TGC	TGC	CCA	GCG	CTG
	CTC	CCA	CCA	CGA	TTC	TTC	CAG	TAG	TAG	AGG	ACG	ACG	GGT	CGC	GAC
91	ACC	CAT	GTT	CGT	CGT	TGG	TGT	CAA	CCT	TGA	AGC	AGT	ATG	ACC	CCT
	TGG	GTA	CAA	GCA	GCA	ACC	ACA	GTT	GGA	ACT	TCG	TCA	TAC	TGG	GGA
136	CTT	ACA	AGG	TCA	TCT	CCA	ACG	CCT	CCT	GCA	CAA	CCA	ACT	GCC	TCG
	GAA	TGT	TCC	AGT	AGA	GGT	TGC	GGA	GGA	CGT	GTT	GGT	TGA	CGG	AGC
181	CTC	CTC	TCG	CTA	AGG	TCA	TCC	ATG	ACA	ACT	TCG	AGA	TCA	TTG	AAG
	GAG	GAG	AGC	GAT	TCC	AGT	AGG	TAC	TGT	TGA	AGC	TCT	AGT	AAC	TTC
226	GTC	TGA	TGA	CCA	CTG	TAC	ACG	CCA	CCA	CTG	CCA	CCC	AGA	AGA	CAG
	CAG	ACT	ACT	GGT	GAC	ATG	TGC	GGT	GGT	GAC	GGT	GGG	TCT	TCT	GTC
271	TGG	ATG	GAC	CCT	CTG	GTA	AAC	TGT	GGC	GTG	ATG	GCC	GTG	GTG	CTC
	ACC	TAC	CTG	GGA	GAC	CAT	TTG	ACA	CCG	CAC	TAC	CGG	CAC	CAC	GAG
316	AGC	AGA	ATA	TCA	TTC	CCG	CGG	AAT	TCC	CCA	GCC	GCA	GCT	AGC	TAA
	TCG	TCT	TAT	AGT	AAG	GGC	GCC	TTA	AGG	GGT	CGG	CGT	CGA	TCG	ATT

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Fig.2 i.

361 CCT GCA GCA GAC ACA ACC CCT ACC TTC CAT GCC GTT ACC AAT GCC
 GGA CGT CGT CGT CTG TGT TGG GGA TGG AAG GTA CGG CAA TGG TTA CGG

 406 ACC GAC AAC ACC CAA ATC AGA AAA CGA GTC AAT GTC ATC AGG TCG
 TGG CTG TTG TGG GTT TAG TCT TTT GCT CAG TTA CAG TAG TCC AGC

 451 TGA GGA ACT GTC TCC AGC TTC GAG TGT AAA CGG CTG CAG CAC AGA
 ACT CCT TGA CAG AGG TCG AAG CTC ACA TTT GCC GAC GTC GTG TCT

 496 TGG CGA GGC GAG GCG GCA GAA GAA AGG CCC AGC GCC GAG GCA GCA
 ACC GCT CCG CTC CGC CGT CTT CTT TCC GGG TCG CGG CTC CGT CGT

 541 AGA AGA GCT ATG TCT TGT CTG CGG CGA CAG AGC CTC CGG ATA TCA
 TCT TCT CGA TAC AGA ACA GAC GCC GCT GTC TCG GAG GCC TAT AGT

 586 CTA CAA CGC GCT CAC ATG TGA AGG GTG TAA AGG TTT CTT CAG GCG
 GAT GTT GCG CGA GTG TAC ACT TCC CAC ATT TCC AAA GAA GTC CGC

 631 GAG TGT AAC CAA AAA TGC AGT GTA CAT ATG CAA ATT CGG CCA TGC
 CTC ACA TTG GTT TTT ACG TCA CAT GTA TAC GTT TAA GCC GGT ACG

 676 TTG CGA AAT GGA TAT CTA TAT GCG GAG AAA ATG TCA GGA GTG TCG
 AAC GCT TTA CCT ATA GAT ATA CGC CTC TTT TAC AGT CCT CAC AGC

 721 GTT GAA GAA ATG TCT TGC GGT GGG CAT GAG GCC CGA GTG CGT GGT
 CAA CTT CTT TAC AGA ACG CCA CCC GTA CTC CGG GCT CAC GCA CCA

 766 GCC GGA GAA CCA GTG TGC AAT GAA ACG GAA AGA GAA AAA GGC GCA
 CGG CCT CTT GGT CAC ACG TTA CTT TGC CTT TCT TCT TTT CCG CGT

4/56

Fig.2 ii.

811 GAG GGA AAA AGA CAA ATT GCC CGT CAG TAC GAC GAC AGT AGA CGA
 CTC CCT TTT TCT TAA CGG GCA GTC ATG CTG CTG TCA TCT GCT

856 TCA CAT GCC TCC CAT CAT GCA ATG TGA CCC TCC GCC CCC AGA GGC
 AGT GTA CGG AGG GTA GTA CGT TAC ACT GGG AGG CGG GGG TCT CCG

901 CGC TAG AAT TCT GGA ATG TGT GCA GCA GGT GGT GCC ACG ATT
 GCG ATC TTA AGA CCT TAC ACA CGT CGT CCA CCA CGG TGC TAA

946 CCT GAA TGA GAA GCT AAT GGA ACA GAA CAG ATT GAA GAA CGT GCC
 GGA CTT ACT CTT CGA TTA CCT TGT CTT GTC TAA CTT CTT GCA CGG

991 CCC CCT CAC TGC CAA TCA GAA GTC GTT GAT CGC AAG GCT CGT GTG
 GGG GGA GTG ACG GTT AGT CTT CAG CAA CTA GCG TTC CGA GCA CAC

1036 GTA CCA GGA AGG CTA TGA ACA ACC TTC CGA GGA AGA CCT GAA GAG
 CAT GGT CCT TCC GAT ACT TGT TGG AAG GCT CCT TCT GGA CTT CTC

1081 GGT TAC ACA GTC GGA CGA GGA CGA AGA CTC GGA TAT GCC GTT
 CCA ATG TGT CAG CCT GCT CCT GCT TCT GAG CCT ATA CGG CAA

1126 CCG TCA GAT TAC CGA GAT GAC GAT TCT CAC AGT GCA GCT CAT CGT
 GGC AGT CTA ATG GCT CTA CTG CTA AGA GTG TCA CGT CGA GTA GCA

1171 AGA ATT CGC TAA GGG CCT CCC GGG CTT CGC CAA GAT CTC GCA GTC
 TCT TAA GCG ATT CCC GGA GGG CCC GAA GCG GTT CTA GAG CGT CAG

1216 GGA CCA GAT CAC GTT ATT AAA GGC GTG CTC AAG TGA GGT GAT GAT
 CCT GGT CTA GTG CAA TAA TTT CCG CAC GAG TTC ACT CCA CTA CTA

1261 GCT CCG AGT GGC TCG GCG GTA TGA CGC GGC CAC CGA CAG CGT ACT
 CGA GGC TCA CCG AGC CGC CAT ACT GCG CCG GTG GCT GTC GCA TGA

5/56

Fig.2 iii.

1306 GTT CGC GAA CAA CCA GGC GTA CAC TCG CGA CAA CTA CCG CAA GGC
 CAA GCG CTT GTT GGT CCG CAT GTG AGC GCT GTT GAT GGC GTT CCG

 1351 AGG CAT GGC GTA CGT CAT CGA GGA CCT GCT GCA CTT CTG TCG GTG
 TCC GTA CCG CAT GCA GTA GCT CCT GGA CGA CGT GAA GAC AGC CAC

 1396 CAT GTA CTC CAT GAT GAT GGA TAA CGT GCA TTA TGC GCT GCT TAC
 GTA CAT GAG GTA CTA CTA CCT ATT GCA CGT AAT ACG CGA CGA ATG

 1441 AGC CAT TGT CAT CTT CTC AGA CCG GCC CGG GCT TGA GCA ACC CCT
 TCG GTA ACA GTA GAA GAG TCT GGC CGG GCC CGA ACT CGT TGG GGA

 1486 GTT GGT GGA GGA CAT CCA GAG ATA TTA CCT GAA CAC GCT ACG GGT
 CAA CCA CCT CCT GTA GGT CTC TAT AAT GGA CTT GTG CGA TGC CCA

 1531 GTA CAT CCT GAA CCA GAA CAG CGC GTC GCC CGG CGC CGT CAT
 CAT GTA GGA CTT GGT CTT GTC GCG CAG CGG GGC GCC GCG GCA GTA

 1576 CTT CGG CGA GAT CCT GGG CAT ACT GAC GGA GAT CCG CAC GCT GGG
 GAA GCC GCT CTA GGA CCC GTA TGA CTG CCT CTA GGC GTG CGA CCC

 1621 CAT GCA GAA CTC CAA CAT GTG CAT CTC CCT CAA GCT GAA GAA CAG
 GTA CGT CTT GAG GTT GTA CAC GTA GAG GGA GTT CGA CTT CTT GTC

 1666 GAA GCT GCC GCG GTT CCT CGA GGA GAT CTG GGA CGT GGC GGA CGT
 CTT CGA CCG CGG CAA GGA GCT CCT CTA GAC CCT GCA CCG CCT GCA

 1711 GGC GAC GAC GGC GAC GCC GGT GGC GGC GGA GGC GCC GCC TCT
 CCG CTG CTG CCG CTG CCG CCA CCG CCG CCT CCG CGG CCG CGG AGA

6/56

Fig.2 iv.

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1756 AGC CCC CGC CCC GCC CGC CCG GCC CGC CAC CGT CTA GCG GCG CGC
TCG GGG GCG GGG CGG CGG GGC CGC CGG CGG GCG GAT CGC GCG

1801 CTC AGG AGA GAA CGC TCA TAG ACT GGC TAG TTT TAG TGA AGT GCA
GAG TCC TCT CTT GCG AGT ATC TGA CCG ATC AAA ATC ACT TCA CGT

1846 CGG ACA CTG ACG TCG ACG TGA TCA ACC TAT TTA TAA GGA CTG CGA
GCC TGT GAC TGC AGC TGC ACT AGT TGG ATA AAT ATT CCT GAC GCT

1891 ATT TTA CCA CTT AAG AGG GCA CAC CCG TAC CCG ATT TCG TAC GG
TAA AAT GGT GAA TTC TCC CGT GTG GGC ATG GGC TAA AGC ATG CC

```

Total number of bases is: 1934.

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The sequence shown below is that of pSK16.1

	3	9	15	21	27	33	39	45
1	CGC TGG TAT AAC AAC GGA CCA TTC CAG ACG CTG CGA ATG CTC GAG GCG ACC ATA TTG TTG CCT GGT AAG GTC TGC AGT TCA GCA CTG GGC CTG GAC CTC							
46	GAG AGC TCG TCT GAG GTG ACG TCG TCT TCA GCA CTG GGC CTG CCG CTC TCG AGC AGA CTC CAC TGC AGC AGA AGT CGT GAC CCG GAC GGC							
91	CCG GCT ATG GTG ATG TCC CCG GAA TCG CTC GCG TCG CCC GAG ATC GGC CGA TAC CAC TAC AGG GGC CTT AGC GAG CGC AGC GGC CTC TAG							
136	GGC GGC CTG GAG CTG TGG GGC TAC GAC GAT GGC ATC ACT TAC AGC CCG CCG GAC CTC GAC ACC CCG CTG CTG CTA CCG TAG TGA ATG TCG							
181	ATG GCA CAG TCG CTG GGC ACC TGC ACC ATG GAG CAG CAG CCC TAC CGT GTC AGC TCG GAC CCG TGG ACC TGG TAC CTC GTC GTC GGC							

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Fig.3 i.

226 CAG CCG CAG CAG CAG CCG CAG CAG ACA CAA CCC CTA CCT TCC ATG
 GTC GGC GTC GTC GTC GTC GTC TGT GGT GGG GAT GGA AGG TAC

 271 CCG TTA CCA ATG CCA CCG ACA ACA CCC AAA TCA GAA AAC GAG TCA
 GGC AAT GGT TAC GGT GGC TGT TGT GGT TTT AGT CTT TTG CTC AGT

 316 ATG TCA TCA GGT CGT GAG GAA CTG TCT CCA GCT TCG AGT GTA AAC
 TAC AGT AGT CCA GCA CTC CTT GAC AGA GGT CGA AGC TCA CAT TTG

 361 GGC TGC AGC ACA GAT GGC GAG GCG AGG CCG CAG AAG AAA GGC CCA
 CCG ACG TCG TGT CTA CCG CTC CGC TCC GCC GTC TTC TTT CCG GGT

 406 GCG CCG CCG AGG CAG CAA GAA GAG CTA TGT CTT GTC TGC GGC GAC AGA
 CCG GGC TCC GTC GTC GTC CTT CTT GAT ACA GAA CAG ACG CCG CTG TCT

 451 GCC TCC GGA TAT CAC TAC AAC GCG CTC ACA TGT GAA GGG TGT AAA
 CCG AGG CCT ATA GTG ATG TTG CGC GAG TGT ACA CTT CCC ACA TTT

 496 GGT TTC TTC AGG CCG AGT GTA ACC AAA AAT GCA GTG TAC ATA TGC

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Fig.3 ii.

CCA AAG AAG TCC GCC TCA CAT TGG TTT TTA CGT CAC ATG TAT ACG

541 AAA TTC GGC CAT GCT TGC GAA ATG GAT ATC TAT ATG CCG AGA AAA
TTT AAG CCG GTA CGA ACG CTT TAC CTA TAG ATA TAC GCC TCT TTT

586 TGT CAG GAG TGT CGG TTG AAG AAA TGT CTT GCG GTG GGC ATG AGG
ACA GTC CTC ACA GCC AAC TTC TTT ACA GAA CGC CAC CCG TAC TCC

631 CCC GAG TGC GTG GTG CCG GAG AAC CAG TGT GCA ATG AAA CCG AAA
GGG CTC ACG CAC CAC CAC GGC CTC TTG GTC ACA CGT TAC TTT GCC TTT

676 GAG AAA AAG GCG CAG AGG GAA AAA GAC AAA TTG CCC GTC AGT ACG
CTC TTT TTC CGC GTC TCC CTT TTT CTG TTT AAC GGG CAG TCA TGC

721 ACG ACA GTA GAC GAT CAC ATG CCT CCC ATC ATG CAA TGT GAC CCT
TGC TGT CAT CTG CTA GTG TAC GGA GGG TAG TAC GTT ACA CTG GGA

766 CCG CCC CCA GAG GCC GCT AGA ATT CTG GAA TGT GTG CAG CAC GAG
GGC GGG GGT CTC CCG CGA TCT TAA GAC CTT ACA CAC GTC GTG CTC

811 GTG GTG CCA CGA TTC CTG AAT GAG AAG CTA ATG GAA CAG AAC AGA
CAC CAC GGT GCT AAG GAC TTA CTC TTC GAT TAC CTT GTC TTG TCT

856 TTG AAG AAC GTG CCC CCC CTC ACT GCC AAT CAG AAG TCG TTG ATC
AAC TTC TTG CAC GGG GGG GAG TGA CCG TTA GTC TTC AGC AAC TAG

901 GCA AGG CTC GTG TGG TAC CAG GAA GGC TAT GAA CAA CCT TCC GAG
CGT TCC GAG CAC ACC ATG GTC CTT CCG ATA CTT GTT GGA AGG CTC

946 GAA GAC CTG AAG AGG GTT ACA CAG TCG GAC GAG GAC GAA GAC
CTT CTG GAC TTC TCC CAA TGT GTC AGC CTG CTC CTG CTT CTG

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Fig.3 iii.

991 TCG GAT ATG CCG TTC CGT CAG ATT ACC GAG ATG ACG ATT CTC ACA
 AGC CTA TAC GGC AAG GCA GTC TAA TGG CTC TAC TGC TAA GAG TGT

 1036 GTG CAG CTC ATC GTA GAA TTC GCT AAG GGC CTC CCG GGC TTC GCC
 CAC GTC GAG TAG CAT CTT AAG CGA TTC CCG GAG GGC CCG AAG CGG

 1081 AAG ATC TCG CAG TCG GAC CAG ATC ACG TTA TTA AAG GCG TGC TCA
 TTC TAG AGC GTC AGC CTG GTC TAG TGC AAT AAT TTC CGC ACG AGT

 1126 AGT GAG GTG ATG ATG CTC CGA GTG GCT CGG CGG TAT GAC GCG GCC
 TCA CTC CAC TAC TAC GAG GCT CAC CGA GCC ATA CTG CGC CGG

 1171 ACC GAC AGC GTA CTG TTC GCG AAC AAC CAG GCG TAC ACT CGC GAC
 TGG CTG TCG CAT GAC AAG CGC TTG TTG GTC CGC ATG TGA GCG CTG

 1216 AAC TAC CGC AAG GCA GGC ATG GCG TAC GTC ATC GAG GAC CTG CTG
 TTG ATG GCG TTC CGT CCG TAC CGC ATG CAG TAG CTC CTG GAC GAC

 1261 CAC TTC TGT CGG TGC ATG TAC TCC ATG ATG ATG GAT AAC GTG CAT
 GTG AAG ACA GCC ACG TAC ATG AGG TAC TAC TAC CTA TTG CAC GTA

 1306 TAT GCG CTG CTT ACA GCC ATT GTC ATC TTC TCA GAC CGG CCC GGG
 ATA CGC GAC GAA TGT CGG TAA CAG TAG AAG AGT CTG GCC GGG CCC

 1351 CTT GAG CAA CCC CTG TTG GTG GAG GAC ATC CAG AGA TAT TAC CTG
 GAA CTC GTT GGG GAC AAC CAC CTC CTG TAG GTC TCT ATA ATG GAC

 1396 AAC ACG CTA CGG GTG TAC ATC CTG AAC CAG AAC AGC GCG TCG CCC
 TTG TGC GAT GCC CAC ATG TAG GAC TTG GTC TTG TCG CGC AGC GGG

 1441 CGC GGC GCC GTC ATC TTC GGC GAG ATC CTG GGC ATA CTG ACG GAG

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Fig.3 iv.

GCG CCG CCG CAG TAG AAG CCG CTC TAG GAC CCG TAT GAC TGC CTC
 1486 ATC CGC ACG CTG GGC ATG CAG AAC TCC AAC ATG TGC ATC TCC CTC
 TAG GCG TGC GAC CCG TAC TCG GTC TCG AAC TCG TAC ACG TAG AGG GAG
 1531 AAG CTG AAG AAC AGG AAG CTG CCG CCG TTC CTC GAG GAG ATC TGG
 TTC GAC TTC TTG TCC TTC GAC GGC AAC GAG CTC CTC TAG ACC
 1576 GAC GTG GCG GAC GTG GCG ACG ACG CCG GTG GCG GCG GAG
 CTG CAC CGC CTG CAC CGC TGC TGC CGC TGC GGC CAC CGC CGC CTC
 1621 GCG CCG GCG CCT CTA GCC CCC GCG CCC GCG CCG CCC GCC
 CGC GCG CGC GCG GAT CGG GCG GCG GCG GCG GCG GCG GCG GCG
 1666 ACC GTC TAG CGC GCC TCA GGA GAG AAC GCT CAT AGA CTG GCT AGT
 TGG CAG ATC GCG CGG AGT CCT CTC TTG CGA GTA TCT GAC CGA TCA
 1711 TTT AGT GAA GTG CAC GGA CAC TGA CGT CGA CGT GAT CAA CCT ATT
 AAA TCA CTT CAC GTG CCT GTG ACT GCA GCT GCA CTA GTT GGA TAA
 1756 TAT AAG GAC TGC GAA TTT TAC CAC TTA AGA GGG CAC ACC CGT ACC
 ATA TTC CTG ACG CTT AAA ATG GTG AAT TCT CCC GTG TGG GCA TGG
 1801 CGA TTT CGT ACG TAT TCG GTG ACC GAC GAC GAT GCA GAG CGT GTG
 GCT AAA GCA TGC ATA AGC CAC TGG CTG CTG CTA CGT CTC GCA CAC
 1846 TAA TGT GAA TAT ATG TGT TGT TGA ACG ATT TGG AGA ATA TAT ATT
 ATT ACA CTT ATA TAC ACA ACT TGC TAA ACC TCT TAT ATA TAA
 1891 GGT GTT GCT GTT CGG GCC CGC ACG CCG TCG CCG GTC GGC GGC GAT
 CCA CAA CGA CAA GCC CGG GCG TGC GCG AGC GGC CAG CCG CCG CTA

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Fig.3 v.

1936 CGC GGC GCC CGC GGC TTC AGT TTT ATT TCG TTT ACG ACT GAG TTG
 GCG CCG CGG GCG CCG AAG TCA AAA TAA AGC AAA TGC TGA CTC AAC

 1981 GTC ACT CGG ATA CGA CTG TAT GAT AAG ACT TCG TTC GAT AAG TAC
 CAG TGA GCC TAT GCT GAC ATA CTA TTC TGA AGC AAG CTA TTC ATG

 2026 ACC TAC TAA ATT ACA CAT ACG TAC GTA GCT TAC GAG AGT TAT TAG
 TGG ATG ATT TAA TGT GTA TGC ATG CAT CGA ATG CTC TCA ATA ATC

 2071 AGA CAA AGA ATA TAA GAA GAA GAT GTT TCT ATT GGG TGA AAA GTT
 TCT GTT TCT TAT ATT ATT CTT CTA CAA AGA TAA CCC ACT TTT CAA

 2116 GAT AGT TAT GTT TAT TTA CCA AAA TTA ACA ATA ATA CGT TGA TTA
 CTA TCA ATA CAA ATA AAT GGT TTT AAT TGT TAT TAT GCA ACT AAT

 2161 ACC TTT CGA GTA TAA TAT TGT GAT GAG TCG TCC GCT GTC CAC GTC
 TGG AAA GCT CAT ATT ATA ACA CTA CTC AGC AGG CGA CAG GTG CAG

 2206 GCC GTC ACA TGT TTG TTT CTG ATG CAC ACG TGA GGN GCG TTA TCG
 CGG CAG TGT ACA AAC AAA GAC TAC GTG TGC ACT CCN CGC AAT AGC

 2251 TGT TTC ATG GTT CCA TCG TCC TGT GCC CGC GAC CCT CGA CTA AAT
 ACA AAG TAC CAA GGT AGC AGG ACA CGG GCG CTG GGA GCT GAT TTA

 2296 GAG TAA TTT AAT TTA TTG CTG TGA TTA CAT TTT AAT GTG TTG ATT
 CTC ATT AAA TTA AAT AAC GAC ACT AAT GTA AAA TTA CAC AAC TAA

 2341 ATC TAC CAT AGG GTG ATA TAA GTG TGT CTT ATT ACA ATA CAA AGT
 TAG ATG GTA TCC CAC TAT ATT CAC ACA GAA TAA TGT TAT GTT TCA

 2386 GTG TGT CGT CGA TAG CTT CCA CAC GAG CAA GCC TTT TGT TTA AGT

Fig.3 vi.

CAC	ACA	GCA	GCT	ATC	GAA	GGT	GTG	CTC	GTT	CGG	AAA	ACA	AAT	TCA
2431	GAT	TTA	CTG	ACA	TGG	ACA	CTC	GAC	CCG	GAA	CTT	C		
	CTA	AAT	GAC	TGT	ACC	TGT	GAG	CTG	GGC	CTT	GAA	G		

Total number of bases is: 2464.

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Fig.4.

Sequence ID 4

10	20	30	40	50	60
ACTCGCGTGCCTCTCTCACCCTGTTGCTCGGATTGTTGTACTAGAAAAAGTTGTCCGC					
70	80	90	100	110	120
GCTCGAACGAGACTTCCGAGTCCCTATTGGATTGACACGAAAGTCTGAGACAGTGGGATAGCGA					
130	140	150	160	170	180
TTCGGTTTCGTTTGAACGTTGCGGTAGACGAGTGGTGCAATGTCCATGAGTCCGCGTTTAGAT					

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Fig.4 i.

190	200	210	220	230	240
AGTTTAGTGCAGGAAAGTGAAGCCTTCCTCGGAGGATGTCCCTCGGCGCTC					
M S L G A					
250	260	270	280	290	300
GTGGATACCGAGGTGTGACACGGCTCGCCGACATGAGACGCCGCTGGTATAACAACGGAC					
R G Y R R C D T L A D M R R W Y N N G					
310	320	330	340	350	360
CATTCCAGACGCTGCGAATGCTCGAGGAGAGCTCGTCTGAGGTGACGTCGTCTTCAGCAC					
P F Q T L R M L E E S S E V T S S A					
370	380	390	400	410	420
TGGGCCTGCCCGCGCTATGGTGATGTCCCGGAATCGCTCGCGTCGCCCGAGATCGGCG					
L G L P P A M V M S P E S L A S P E I G					

Fig.4 ii.

430 | 440 | 450 | 460 | 470 | 480 |
 GCCTGGAGCTGTGGGGCTACGACGATGGCATCACTTACAGCATGGCACAGTCGCTGGGCA
 G L E L W G Y D D G I T Y S M A Q S L G
 490 | 500 | 510 | 520 | 530 | 540 |
 CCTGCACCATGGAGCAGCAGCAGCCCCAGCCGCGCAGCAGCCGCGCAGCAGACACAACCCC
 T C T M E Q Q Q P Q Q P Q Q Q Q T Q P
 550 | 560 | 570 | 580 | 590 | 600 |
 TACCTTCCATGCCCGTTACCAATGCCACCGACAACACCCAAATCAGAAACGAGTCAATGT
 L P S M P L P M P P T T P K S E N E S M
 610 | 620 | 630 | 640 | 650 | 660 |
 CATCAGGTCGTGAGGAACTGTCTCCAGCTTCCAGTGTAAACGGCTGCAGCACAGATGGCG
 S S G R E E L S P A S S V N G C S T D G
 670 | 680 | 690 | 700 | 710 | 720 |
 AGGCGAGGCGGCAGAAAGGCCCGCCAGCGCCGAGGCAGCAAGAAGAGTATGTCTTGTCT
 E A R R Q K K G P A P R Q Q E E L C L V

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Fig.4 iii.

730 | 740 | 750 | 760 | 770 | 780 |
 GCGGCACAGAGCCTCCGGATATCACTACAACGGCTCACATGTGAAGGGTGTAAAGGTT
 C G D R A S G Y H Y N A L T C E G C K G

 790 | 800 | 810 | 820 | 830 | 840 |
 TCTTCAGCGGAGTGTAACCAAAATGCAGTGACATATGCAAAATTCGGCCATGCTTGCG
 TCTTCAGCGGAGTGTAACCAAAATGCAGTGACATATGCAAAATTCGGCCATGCTTGCG
 F F R R S V T K N A V Y I C K F G H A C

 850 | 860 | 870 | 880 | 890 | 900 |
 AAATGGATATCTATATGCGGAGAAAATGTCAGGAGTGTCGGTTGAAGAAATGTCTTGCGG
 AAATGGATATCTATATGCGGAGAAAATGTCAGGAGTGTCGGTTGAAGAAATGTCTTGCGG
 E M D I Y M R R K C Q E C R L K K C L A

 910 | 920 | 930 | 940 | 950 | 960 |
 TGGGCATGAGGCCCGAGTGCGTGCGGAGAACCAAGTGTGCAATGAAACGGAAAGAGA
 TGGGCATGAGGCCCGAGTGCGTGCGGAGAACCAAGTGTGCAATGAAACGGAAAGAGA
 V G M R P E C V V P E N Q C A M K R K E

 970 | 980 | 990 | 1000 | 1010 | 1020 |
 AAAAGGCGCAGAGGGAAGACAAATGCCCGTCAGTACGACGACAGTAGACGATCACA
 AAAAGGCGCAGAGGGAAGACAAATGCCCGTCAGTACGACGACAGTAGACGATCACA
 K K A Q R E K D K L P V S T T V D D H

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Fig.4 iv.

1030	1040	1050	1060	1070	1080
TGCCCTCCCATGCAATGTGACCCCTCCGCCCCCGAGGCCCTAGAAATCTGTGAATGTG					
M P P I M Q C D P P P E A A R I L E C					
1090	1100	1110	1120	1130	1140
TGCAGCAGGAGGTGGTGCCACGATTCCTGAATGAGAAGCTAATGGAACAGAACAGATTGA					
V Q H E V V P R F L N E K L M E Q N R L					
1150	1160	1170	1180	1190	1200
AGAACGTGCCCCCTCACTGCCAATCAGAAGTCGTTGATCGCAAGGCTCGTGTGTACC					
K N V P P L T A N Q K S L I A R L V W Y					
1210	1220	1230	1240	1250	1260
AGGAAGGCTATGAACAACCTTCGAGGAGAACCTGAAGAGGGTTACACAGTCGGACGAGG					
Q E G Y E Q P S E E D L K R V T Q S D E					

Fig.4 v.

1270 1280 1290 1300 1310 1320
 ACGACGAAGACTCGGATATGCCCGTTCCGTCAGATTACCGAGATGACGATTCTCACAGTGC

D D E D S D M P F R Q I T E M T I L T V

1330 1340 1350 1360 1370 1380
 AGCTCATCGTAGAAATTCGCTAAGGGCCTCCCGGGCTTCGCCCAAGATCTCGCAGTCGGACC

Q L I V E F A K G L P G F A K I S Q S D

1390 1400 1410 1420 1430 1440
 AGATCACGTTATTAAAGCGTGCTCAAGTGAGGTGATGCTCCGAGTGGCTCGGCGGT

Q I T L L K A C S S E V M M L R V A R R

1450 1460 1470 1480 1490 1500
 ATGACGGCGCCACCGACAGCGTACTGTTCGCGAACAACGAGCGGTACACTCGCGACAACCT

Y D A A T D S V L F A N N Q A Y T R D N

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Fig. 4 vi.

1510 1520 1530 1540 1550 1560
 ACCGCAAGGCAGGCATGGCGTACGTACATCGAGGACCTGCTGCACCTTCTGTCTGGTGCATGT
 Y R K A G M A Y V I E D L L H F C R C M
 1570 1580 1590 1600 1610 1620
 ACTCCATGATGATGGATAACGTCATATATGCGCTGCTTACAGCCATTGTTCATCTTCTCAG
 Y S M M D N V H Y A L L T A I V I F S
 1630 1640 1650 1660 1670 1680
 ACCGGCCCGGCTTGAGCAACCCCTGTGTGGAGGAGATCCAGAGATATTACCTGAACA
 D R P G L E Q P L L V E E I Q R Y Y L N
 1690 1700 1710 1720 1730 1740
 CGCTACGGGTGTACATCCTGAACCAAGACGCGCTCGCCCCCGCGCGCGTCACTCTTCG
 T L R V Y I L N Q N S A S P R G A V I F

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Fig.4 vii.

1750 1760 1770 1780 1790 1800
 GCGAGATCCTGGGCATACTGACGGAGATCCGCACGCTGGGCATGCAGAACTCCAACATGT
 G E I L G I L T E I R T L G M Q N S N M
 1810 1820 1830 1840 1850 1860
 GCATCTCCCTCAAGCTGAAGAACAGGAAGCTGCCGCCGTTCTCTCGAGGAGATCTGGGACG
 C I S L K L K N R K L P P F L E E I W D
 1870 1880 1890 1900 1910 1920
 TGGCGGACGTGGCGACGACGGCGACGCCGGTGGCGGGAGGGCGGCCCTCTAGCCC
 V A D V A T T A T P V A A E A P A P L A
 1930 1940 1950 1960 1970 1980
 CCGCCCCCGCCCGCCCGCCCGCCCGTCTAGCGCGCTCAGGAGAGAACGCTCATA
 P A P P A R P P A T V -
 1990 2000 2010 2020 2030 2040
 GACTGGCTAGTTTGTAGTGAAGTGCACGGACACTGACGTGACGTGATCAACCTATTATTA

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Fig.4 viii.

2050 | 2060 | 2070 | 2080 | 2090 | 2100 |
AGGACTGCGAAATTTACCACTTAAGAGGGCACACCCGTACCCGATTTTCGTACGTATTCGG
2110 | 2120 | 2130 | 2140 | 2150 | 2160 |
TGACCGACGACGATGCAGAGCGTGTGTAATGTGAATATATGTGTGTGTTGAACGATTGGA
2170 | 2180 | 2190 | 2200 | 2210 | 2220 |
GAATATATATGGTGTGCTGTTCCGGGCCCCGCACGCCGTCGCCGGCGGATCGCG
2230 | 2240 | 2250 | 2260 | 2270 | 2280 |
GCGCCCGCGGCTTCAGTTTATTTTCGTTTACGACTGAGTTGGTCACTCGGATACGACTGT
2290 | 2300 | 2310 | 2320 | 2330 | 2340 |
ATGATAAGACTTCGTTTCGATAAGTACACCTACTAAATTACACATACGTACGTAGCTTACG
2350 | 2360 | 2370 | 2380 | 2390 | 2400 |
AGAGTTATTAGAGACAAAGAATAATAAGAAGAAGATGTTTCTATTGGGGTGAAAAGTTGATA

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Fig.4 ix.

2410 | 2420 | 2430 | 2440 | 2450 | 2460 |
 GTTATGTTTATTACCAAAATTAACAATAATACGTTGATTAACTTTTCGAGTATAATATT

 2470 | 2480 | 2490 | 2500 | 2510 | 2520 |
 GTGATGAGTCGTCGCTGTCCACGTCGCCGTCACATGTTTGTCTTCTGATGCACACGTGAG

 2530 | 2540 | 2550 | 2560 | 2570 | 2580 |
 GNGCGTTATCGTGTTCATGGTTCCTCCATCGTCCTGTGCCCGGACCCCTCGACTAAATGAGT

 2590 | 2600 | 2610 | 2620 | 2630 | 2640 |
 AATTTAATTTATGCTGTGATTACATTTTAAATGTGTTGATTATCTACCATAGGGTGATAT

 2650 | 2660 | 2670 | 2680 | 2690 | 2700 |
 AAGTGTGCTTATTACAATAACAAGTGTGTGTCGTCGATAGCTTCCACACGAGCAAGCCT

 2710 | 2720 | 2730 | 2740 |
 TTTGTTTAAAGTGATTTACTGACATGGACACTCGACCCGGAATTC

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Fig.5.

Sequence I.D. 5

BmECR	MRVENVDNV/S	10
MseCR	-----	
HvECR	M-----	1
CtECR	-----	
AaECR	-----	
DmECR	-----	

BmECR	FALNGRADEWCMSVETRLDSLVRKSEVKAYVGGCPSVITDAGAYDALFD	60
MseCR	-----	
HvECR	-SLGARGYRRC-----DTLAD	16
CtECR	-----	
AaECR	-----	
DmECR	-----	

BmECR	M-RRRWSNNGGFP-LRMLEESSEVTSSSA-LGLPPAMVMSPELASPEY	107
MseCR	M-RRRWSNNGGFP-LRMFEESSEVTSSSA-FGMPAAMVMSPELASPEY	47
HvECR	M-RRRWYNNGGFQTLRMLEESSEVTSSSA-LGLPPAMVMSPELASPEI	64
CtECR	M-K-----TENLIVTT-VKVEPLNYASQSF	23
AaECR	MMKRRWSNNGGFTALRMLDDSSSEVTSSSAAL-----GMTMSPNSLGSPNY	46
DmECR	M-KRRWSNNGGF--MRLPEESSEVTSSSNGLVLP SGVNMSPSSLDSDHY	47
	* *	

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Fig.5 i.

BmECR	GALELW----	SY-----	114
MsECR	GGLELW----	SY-----	55
HvECR	GGLELW----	GY-----	72
CtECR	GDNNI-----	YGGAT-----	33
AaECR	DELELW-SSYEDNAYNGHSV--	LSNGNNN-----	78
DmECR	CDNDKWLGCNESGSGFSGNGHGLSQQQQSVITLAMHGCSSTLPAQTTIIP		97
BmECR	-----	DDGITY-----	121
MsECR	-----	DETMN-----	61
HvECR	-----	DDGIT-----	77
CtECR	-----	KKQRLESDETMNH-----	46
AaECR	-----	ANLLMNGIVGNL-----	98
DmECR	INGNANGGGSTNGQYVPGATNLGALANGMLNGFGNGMQQIQNGHGLIN		147
BmECR	NTAQSLLGACNMQQQLQP-----	QQHPAPPTLPTMP-----	154
MsECR	YPAQSLLGACNAPQQQQQ-----	QQQPSAQPLPSMP-----	94
HvECR	YSMAQSLGTCMEQQQP-----	QQPQQTQPLPSMP-----	114
CtECR	NQTNMNLESSNMNHTIS-----	GFSSPDVNYEAYSPNSKL-----	86
AaECR	MASQAVQANANSIQHIVGN-----	LINGVNPNTLIPPLPS-----	134
DmECR	STTPSTPTPLHLQNLGGAGGGIGGMGILHANGTPNGLIGVVGGGG		197
BmECR	-----	LPMPTTPKSENESSGREELSPASSINGCSADA--	190
MsECR	-----	LPMPTTPKSENESSGREELSPASSINGCSTDG--	130
HvECR	-----	LPMPTTPKSENESSGREELSPASSVNGCSTDG--	146
CtECR	MSVHMGDG-----	LDG-----	98
AaECR	-----	IIQNTLMNTPRSESVNISSSGREDLSPSSSLNGYT--	173
DmECR	VGLGVGGGVGGLGMQHTPRSDSVNISSSGRDDLSPSSSLNGYSANESCD		247

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Fig.5 ii.

BmECR	ARRQKGPAPRQEEELCLVCGDRASGYHYNALTCEGCKGFFRRSVTKNAV	240
MSECR	PRRQKGPAPRQEEELCLVCGDRASGYHYNALTCEGCKGFFRRSVTKNAV	180
HvECR	ARRQKGPAPRQEEELCLVCGDRASGYHYNALTCEGCKGFFRRSVTKNAV	196
CtECR	KSSSKGVPVRQEEELCLVCGDRASGYHYNALTCEGCKGFFRRSVTKNAV	148
AaECR	AKKQKGPTRQEEELCLVCGDRASGYHYNALTCEGCKGFFRRSVTKNAV	223
DmECR	AKKSKGPPAPRVQEEELCLVCGDRASGYHYNALTCEGCKGFFRRSVTKNAV	297
	**** ** *****	
BmECR	YICKFGHACEMDMYMRKQCERLKKCLAVGMRPECVIEPS-KNKDRQR	289
MSECR	YICKFGHACEMDMYMRKQCERLKKCLAVGMRPECVVPSTCKNKRREK	230
HvECR	YICKFGHACEMDIYMRKQCERLKKCLAVGMRPECVVPENQCAMKRKEK	246
CtECR	YCKKFGHECEMDMYMRKQCERLKKCLAVGMRPECVVPENQCAIKRKEK	198
AaECR	YCKKFGHACEMDMYMRKQCERLKKCLAVGMRPECVVPENQCAIKRKEK	273
DmECR	YCKKFGHACEMDMYMRKQCERLKKCLAVGMRPGCVVPGNQCAMKRREK	347

BmECR	QKKDKGILLPVSTTV-----EDHMPPIMQC	315
MSECR	EAQREKDKLPVSTTV-----DDHMPAIMQC	256
HvECR	KAQREKDKLPVSTTV-----DDHMPPIMQC	272
CtECR	KAQKEKDKVPGIVGSNTSSSLNQSLNNGSLKNLEISYREELLQQLMKC	248
AaECR	KAQKEKDKVQTNAT-----VSTNSTY-RS-----EILPILMKC	306
DmECR	KAQKEKDKMTTSPSSQHGGNGSLASGGQDFVKK-----EILD-LMTC	389

BmECR	DPPPPEAAARI-----HEVVPRYLSEKLMEQNRQKNIPPLSANQKSIAIRL	360
MSECR	DPPPPEAAARI-----HEVVPRLFTEKLMEQNRLLKNVTPLSANQKSIAIRL	301
HvECR	DPPPPEAAARILECVQHEVVPRLFNEKLMEQNRLLKNVPLTANQKSIAIRL	322
CtECR	DPPPHPMQQLL-----PEKLLMENRAKGTPLTANQAVAVIYKL	286
AaECR	DPPPHQAIPLL-----PEKLLQENRLRNIPLLTANQMAVITYKL	344
DmECR	EPQOHATIPLL-----PDEILAKQARNIPSLTYNQLAIVITKL	427

SUBSTITUTE SHEET (RULE 26)

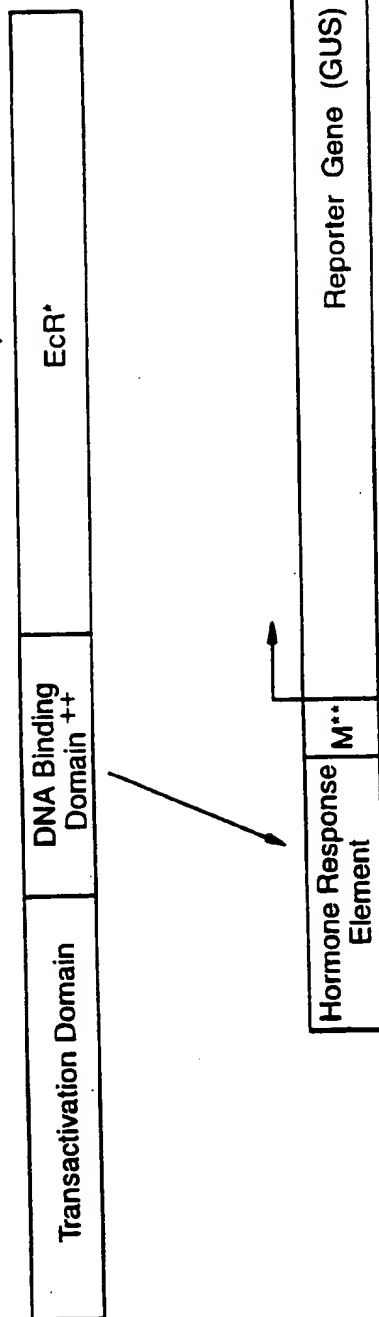
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HvECR	VWYQGYEQPSEEDLKRVTQS---DEDDSDMPFRQITEMTILTVQLI	368
CtECR	IWYQGYEQPSEEDLKRIITTE--LEEEDQEHEANFRYTEVITILTVQLI	334
AaeCR	IWYQGYEQPSEEDLKRIMIG--SPNEEDQHDVHFRHITEITILTVQLI	392
DmeCR	IWYQGYEQPSEEDLRRIM-S-QPDENESQTDVSFRHITEITILTVQLI	474
BmECR	VEFAKGLPGFSKISQSDQITLLKASSSEVMMLRVARRYDAASDVLFANN	459
MSeCR	VEFAKGLPGFSKISQSDQITLLKASSSEVMMLRVARRYDAATDSVLFANN	401
HVeCR	VEFAKGLPGFPAKISQSDQITLLKACSSSEVMMLRVARRYDAATDSVLFANN	418
CteCR	VEFAKGLPAFIKIPQEDQITLLKACSSSEVMMLRMARRYDHSDSILFANN	384
AaeCR	VEFAKGLPAFTKIPOEDQITLLKACSSSEVMMLRMARRYDAATDSILFANN	442
DmeCR	VEFAKGLPAFTKIPOEDQITLLKACSSSEVMMLRMARRYDHSSDISFFANN	524
BmECR	KAYTRDNRYRQGGMAYVIEDLLHFRCRCMFAMGMDNVHFALLTAIVIFSDRP	509
MseCR	QAYTRDNRYRKAGMSYVIEDLLHFRCRCMYSMSMDNVHYALLTAIVIFSDRP	451
HveCR	QAYTRDNRYRKAGMAYVIEDLLHFRCRCMYSMMMDNVHYALLTAIVIFSDRP	468
CteCR	TAYTKQTYQLAGMEETIDDLLHFRCRQMAYSIDNVEALLTAIVIFSDRP	434
AaeCR	RSYTRDSYRMAGMADTIEDLLHFRCRQMFSLTVDNVEYALLTAIVIFSDRP	492
DmeCR	RSYTRDSYKMAGMADNIEDLLHFRCRQFMFKVDNVYALLTAIVIFSDRP	574
BmECR	GLEQPSLVEEIIQRYVLNTLRVIYNQNSASSRCAVIYGRILSVLTTELRTL	559
MseCR	GLEQPLLVEEIIQRYYLKTLRVYILNQHSAPRCVLFKGILGVLTTELRTL	501
HveCR	GLEQPLLVEDIQRYVLNTLRVYILNQNSASPRGAVIFGEILGILTIELRTL	516
CteCR	GLEKAEMVDIIQSYTYTETLKVIVRDHGGERCSVQFAKLGLILTELRTM	48
AaeCR	GLEQAELVEHIQSYVIDTLRIYILNRHAGDPKCSVIFAKLLSILTELRTL	54
DmeCR	GLEKAQLVEAIQSYVIDTLRITILNRHCGBDMSLVFYAKLLSILTELRTL	62

Fig.5 iv.

BmECR	GTQNSNMCISLKLKNRKLPPFLEEIWDVAEVARR-----	593
MsECR	GTQNSNMCISLKLKNRKLPPFLEEIWDVAEVSTT-----	535
HvECR	GMQNSNMCISLKLKNRKLPPFLEEIWDVADVATT-----	552
CtECR	GNLNSMCFSLKLKNRKLPRFLEEVDVGNNTTATTNTENIVRERIN	534
AaECR	GNQNSMCFSLKLKNRKLPRFLEEIWDVQDIPPSMQAQMHSHTQSSS---	590
DmECR	GNQNAEMCFSLKLKNRKLPKFLEEIWDVHAIPPSVQSHLQITQEEDERLE	674
	* * * * * * * * * *	
BmECR	-----	593
MsECR	-----	535
HvECR	-----	552
CtECR	-----	536
AaECR	RN-----	632
DmECR	-----SSSSSSSSNGSSNGSSNSNSHQHPPHPPHQQ--LTPNQ	724
	RAERMRA SVGGAITAGIDCDSASTSAAAAAQHPQHPQPPQPPSSLTQND	
BmECR	-----HPTV-----LPPTNPVVL-----	606
MsECR	-----QP--TPGVAAQVTPIVVDNPAAL-----	556
HvECR	-----ATPVAAEAPAPLAPAPPATV-----	575
CtECR	-----	536
AaECR	QQHQQQ-----HSQLQQ-----V	645
DmECR	SQHQTQPQLQPQLPPQLQGQLQPQLQTLQTLQPQIQPQLLPVSA PV	774
BmECR	-----	606
MsECR	-----	556
HvECR	-----	575
CtECR	-----	536
AaECR	HANGSGSGGSSNNSSSG-----	663
DmECR	PASVTAPGSLSAVSTSEYMGGSAAIGPITPAT TSSITAAVTASSTSAV	824

Fig.6.

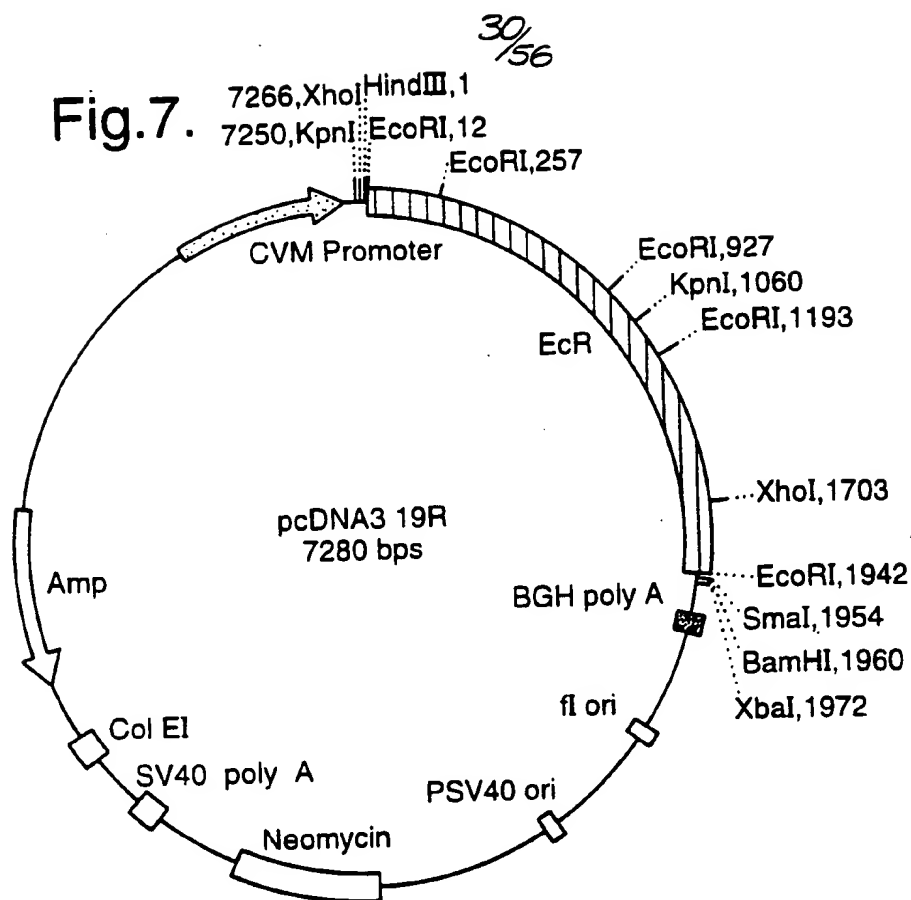
Chemical



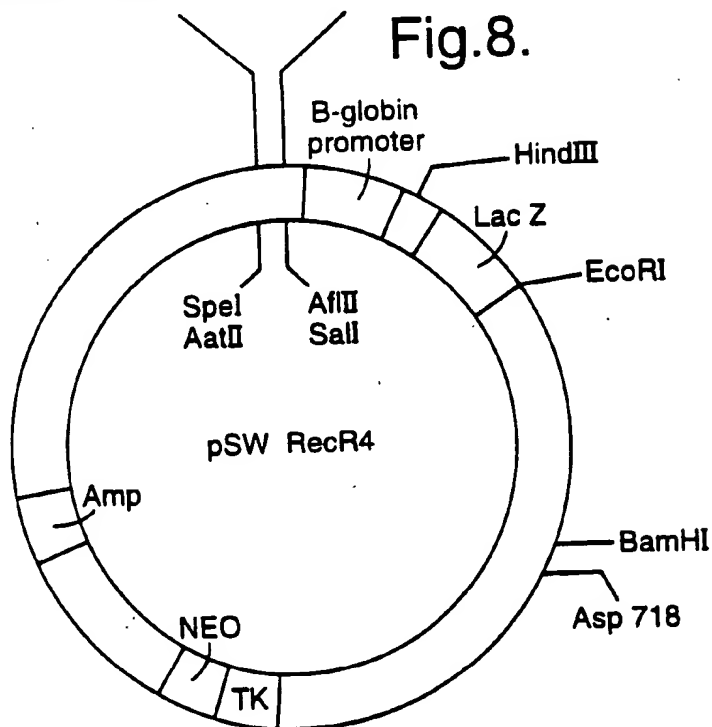
++ Glucocorticoid receptor DNA binding and transactivation domains

* Insect ecdysone ligand binding domain

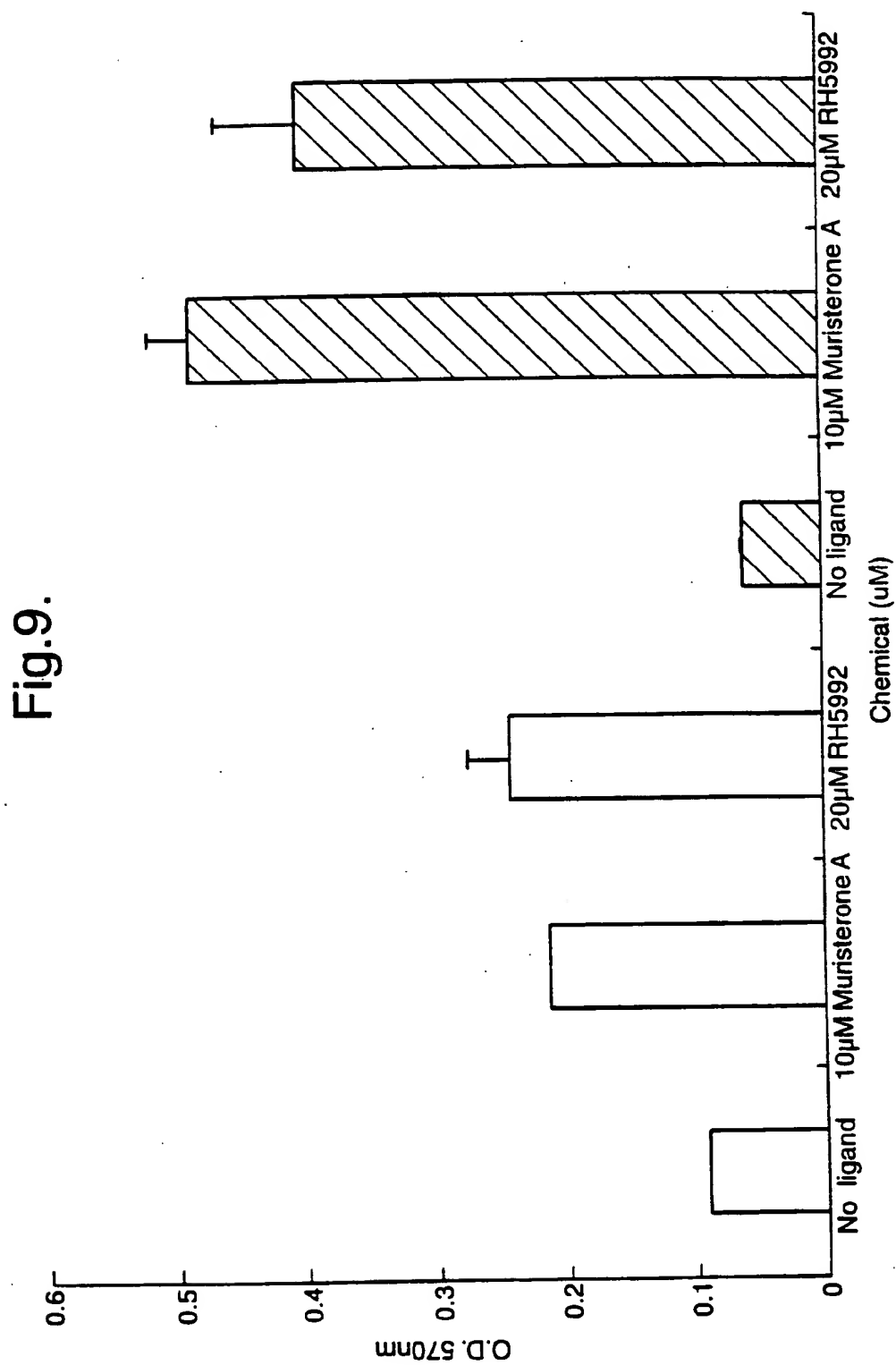
** Minimal 35S CaMV promoter



Response Element for HecR → → →

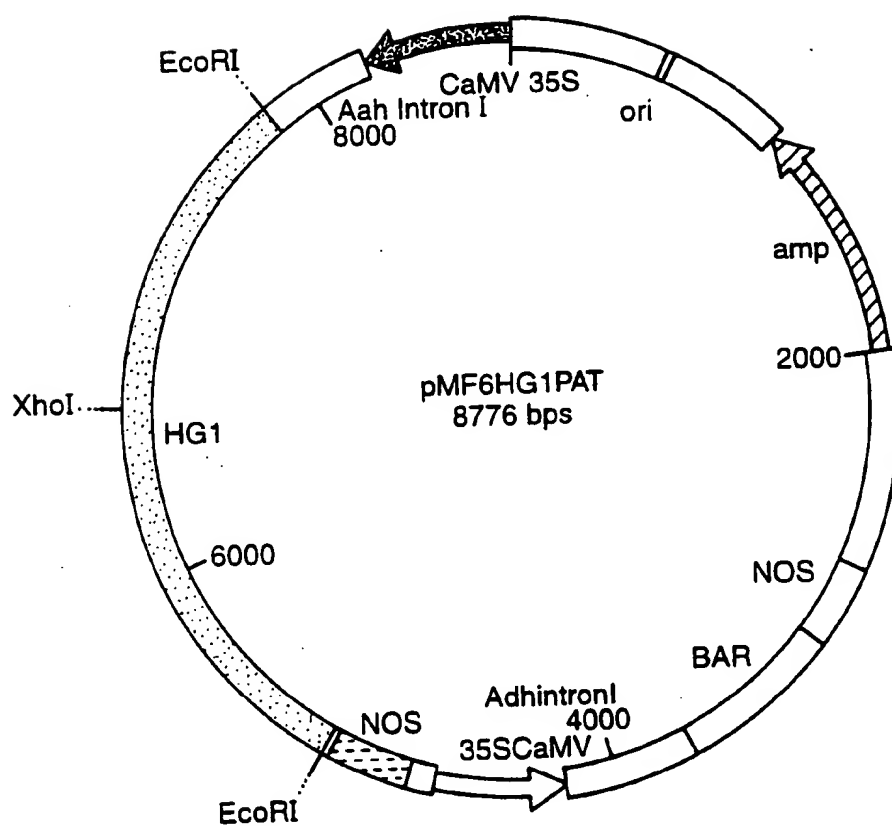


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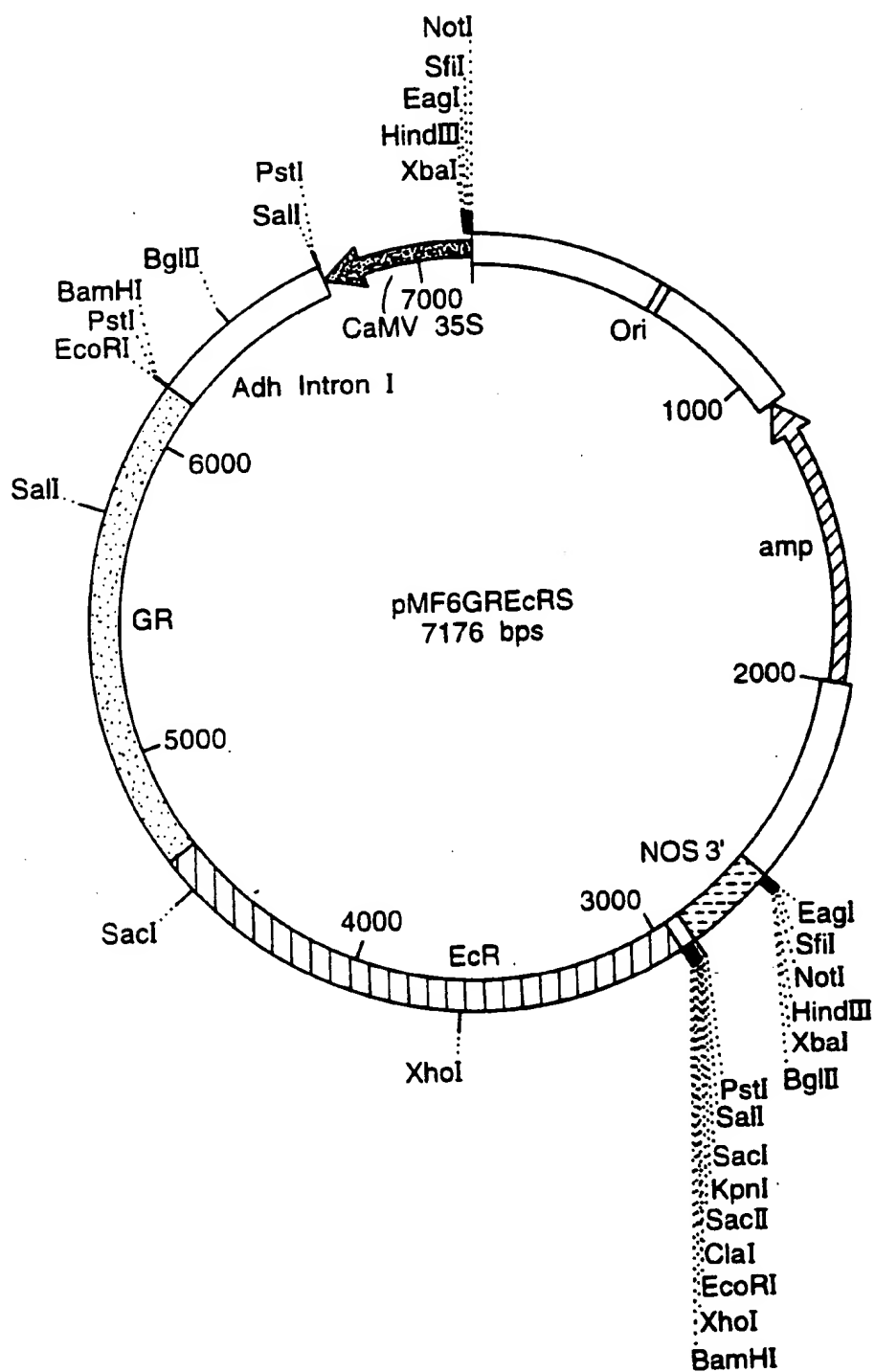
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Fig.10.



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Fig.11.



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Fig.12.

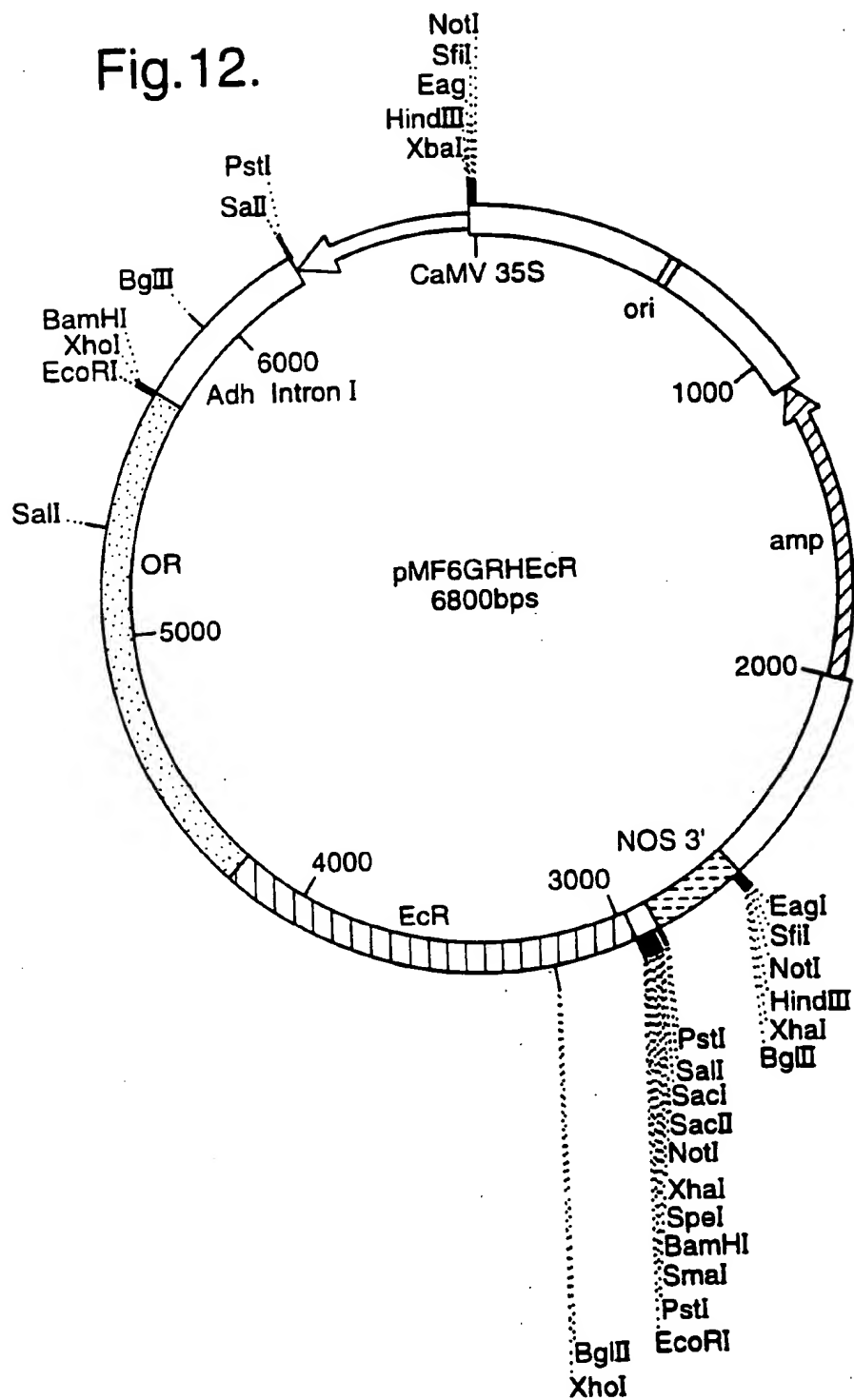


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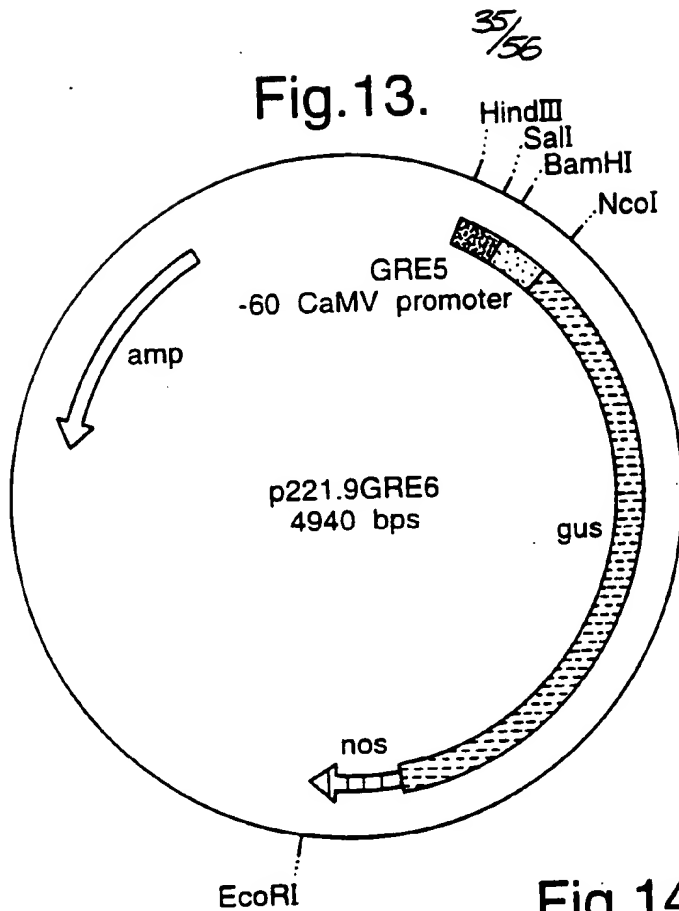
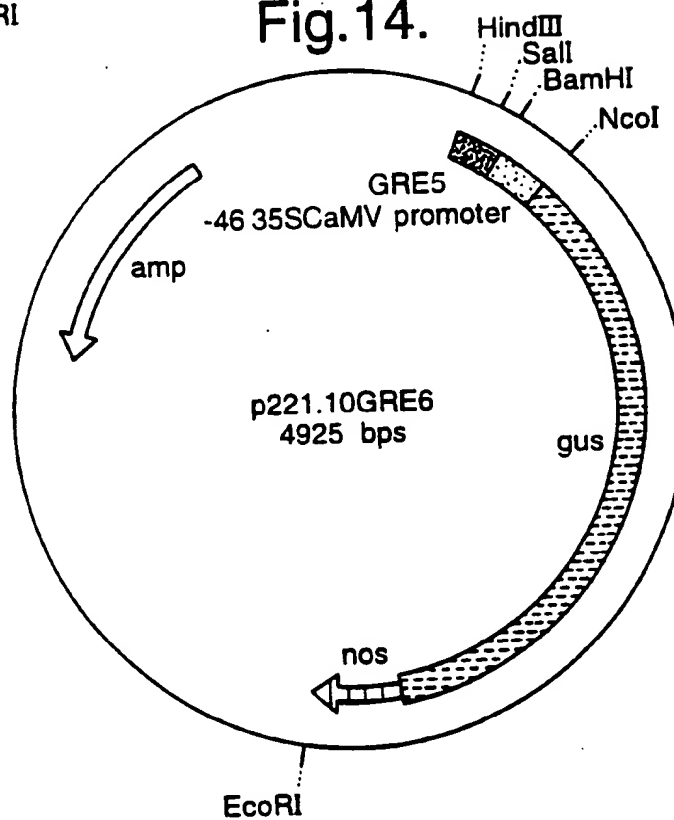


Fig.14.



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Fig.15.

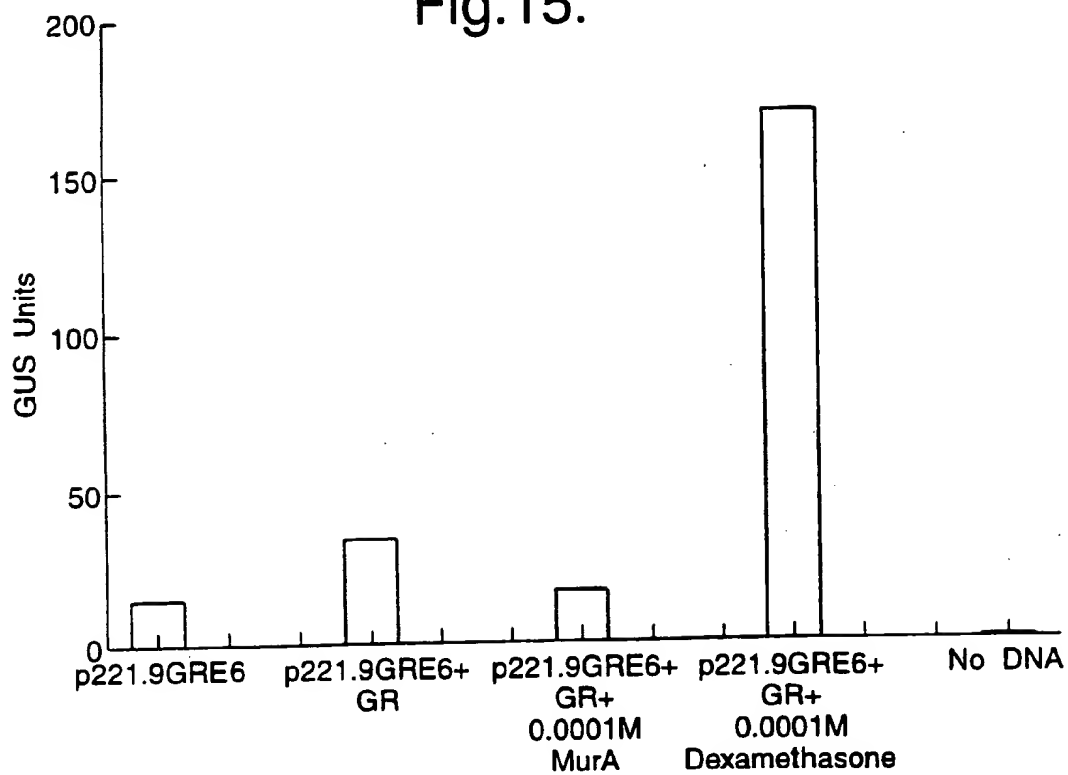
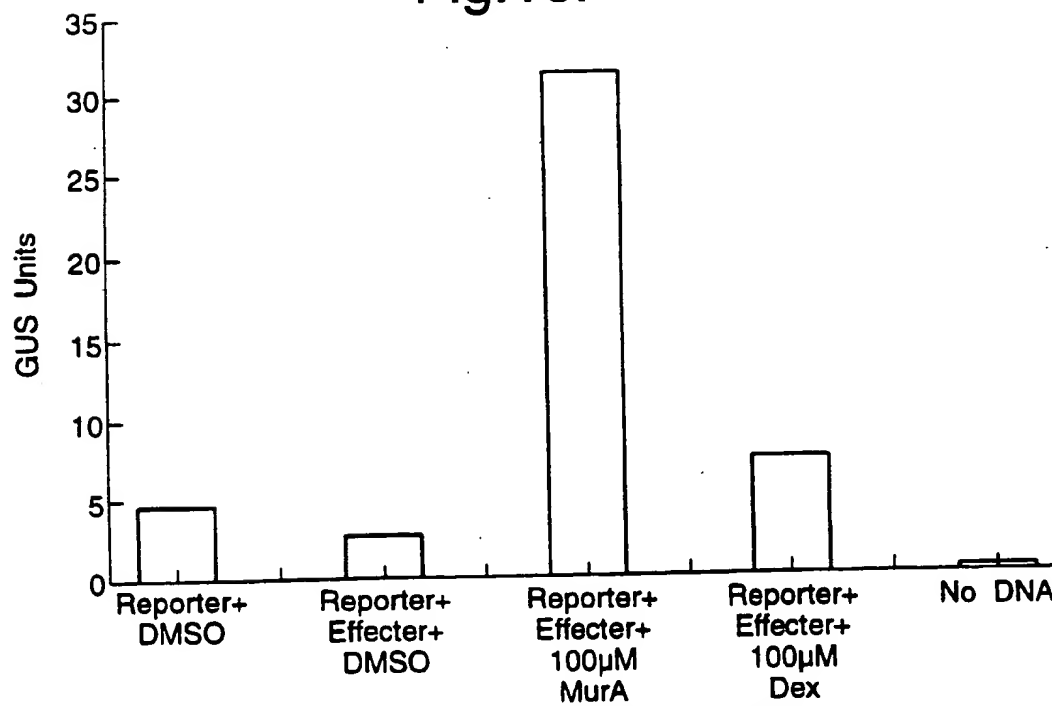


Fig.16.



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Fig.17.

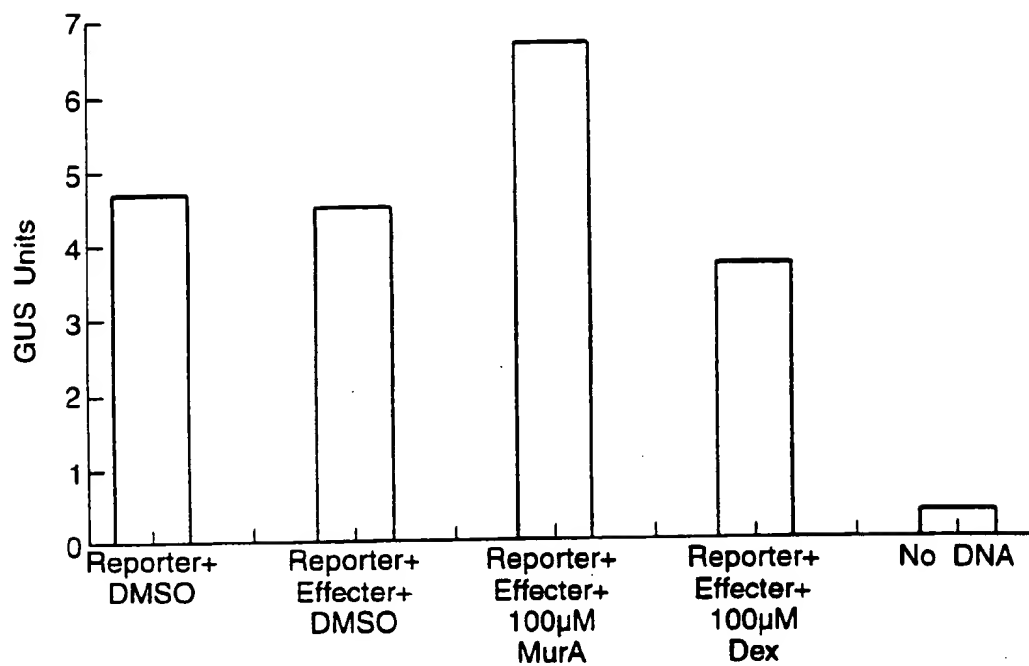
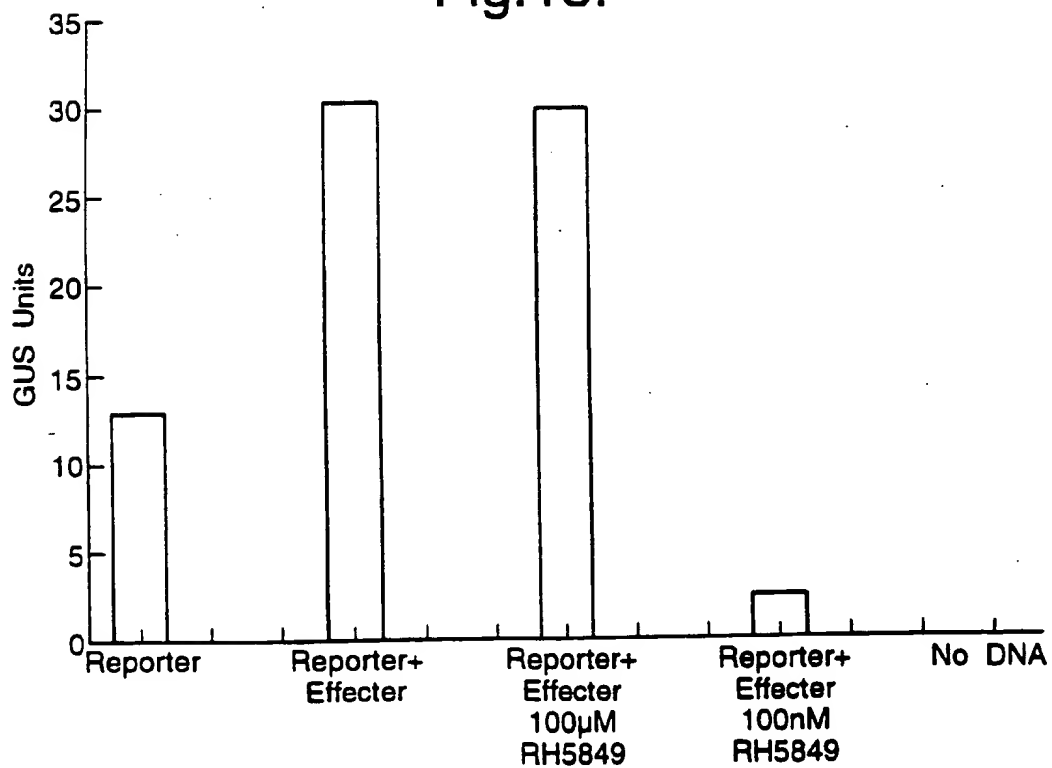


Fig.18.



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Fig.19.

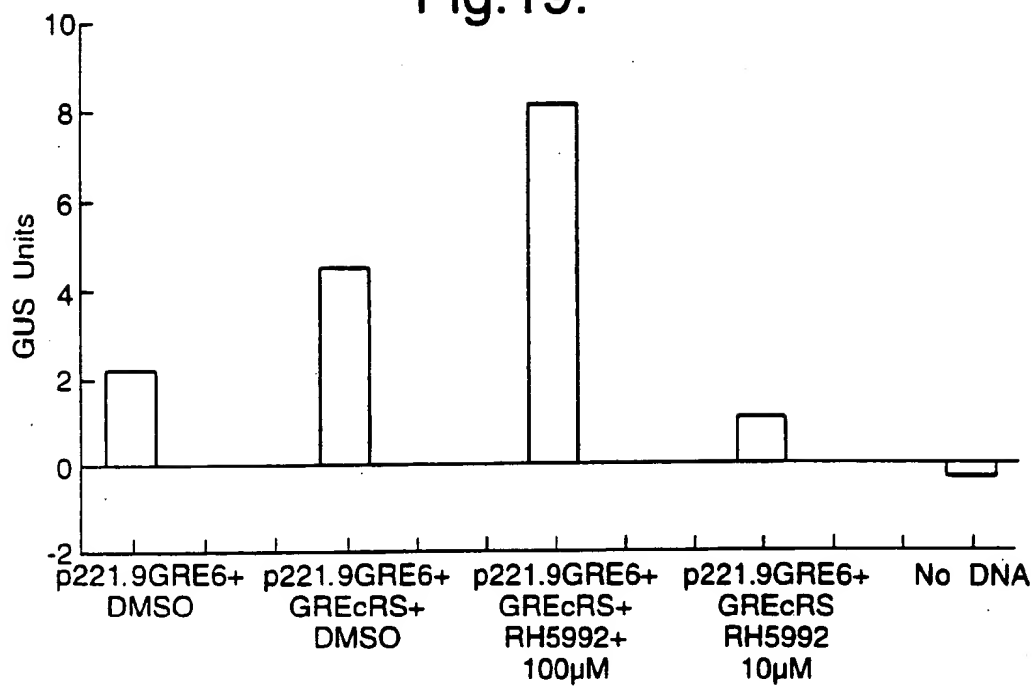
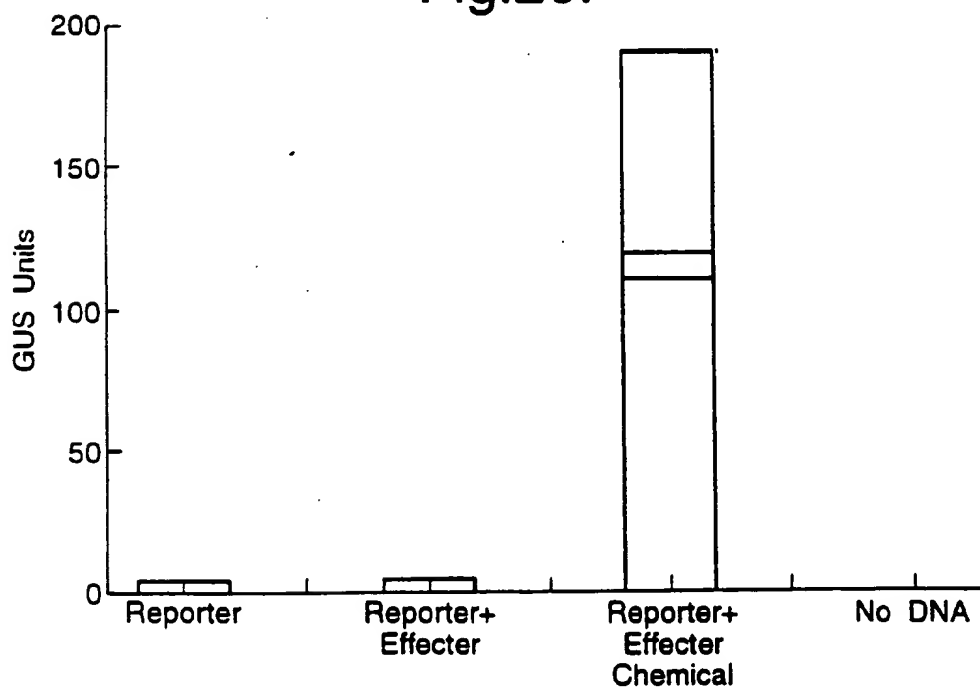
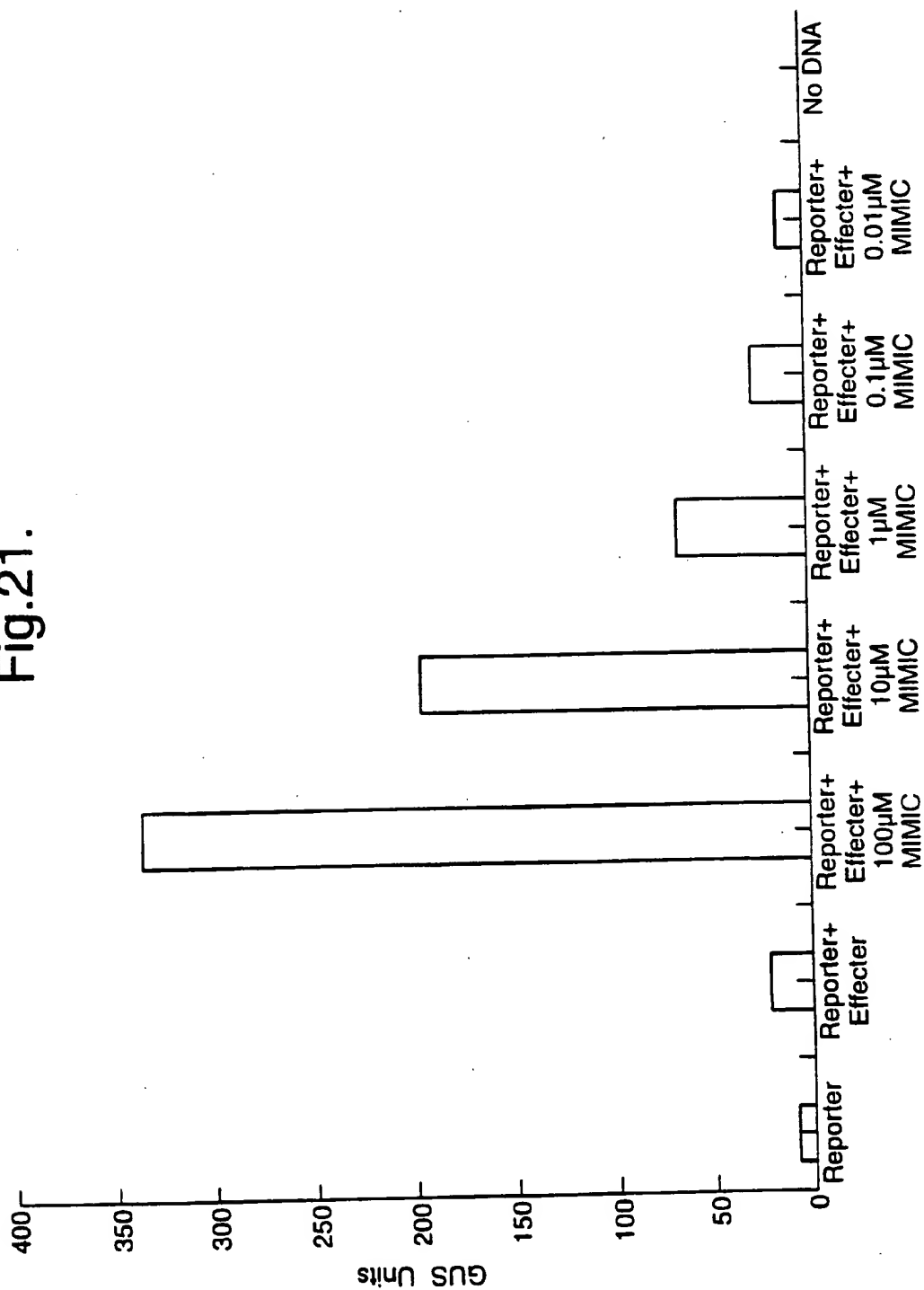


Fig.20.



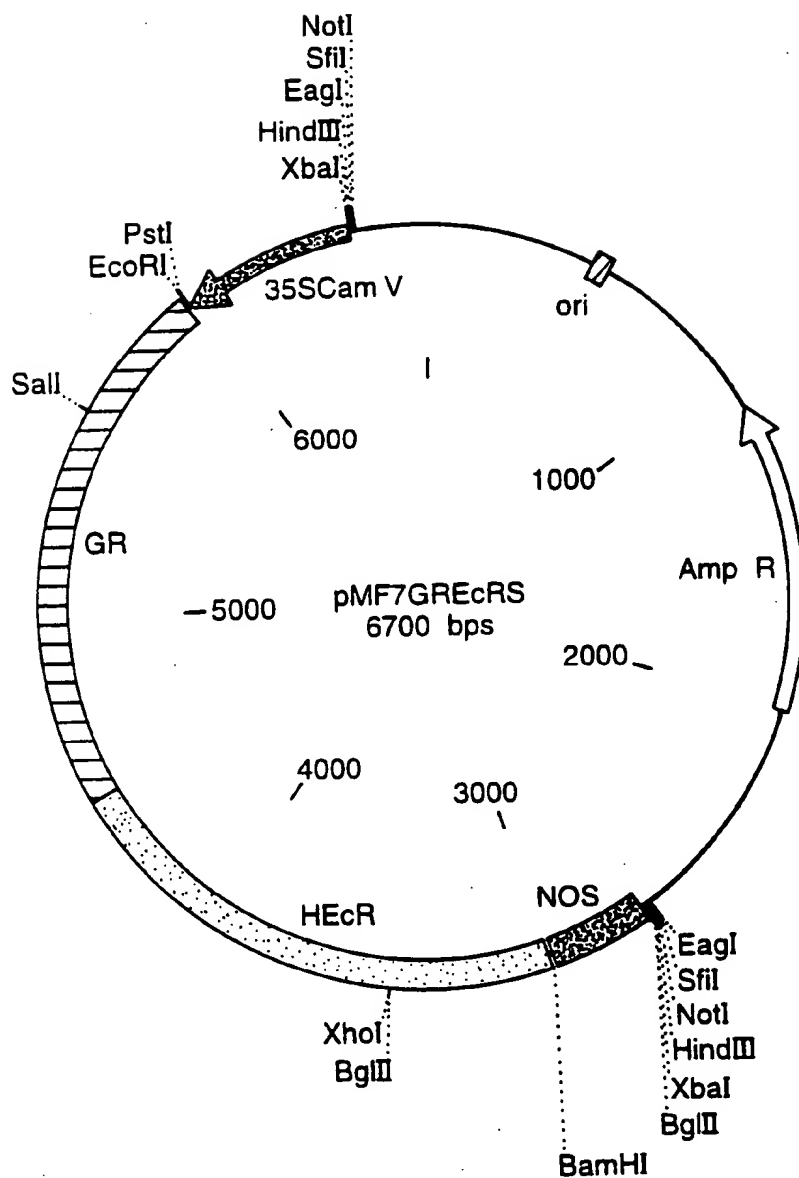
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Fig.21.



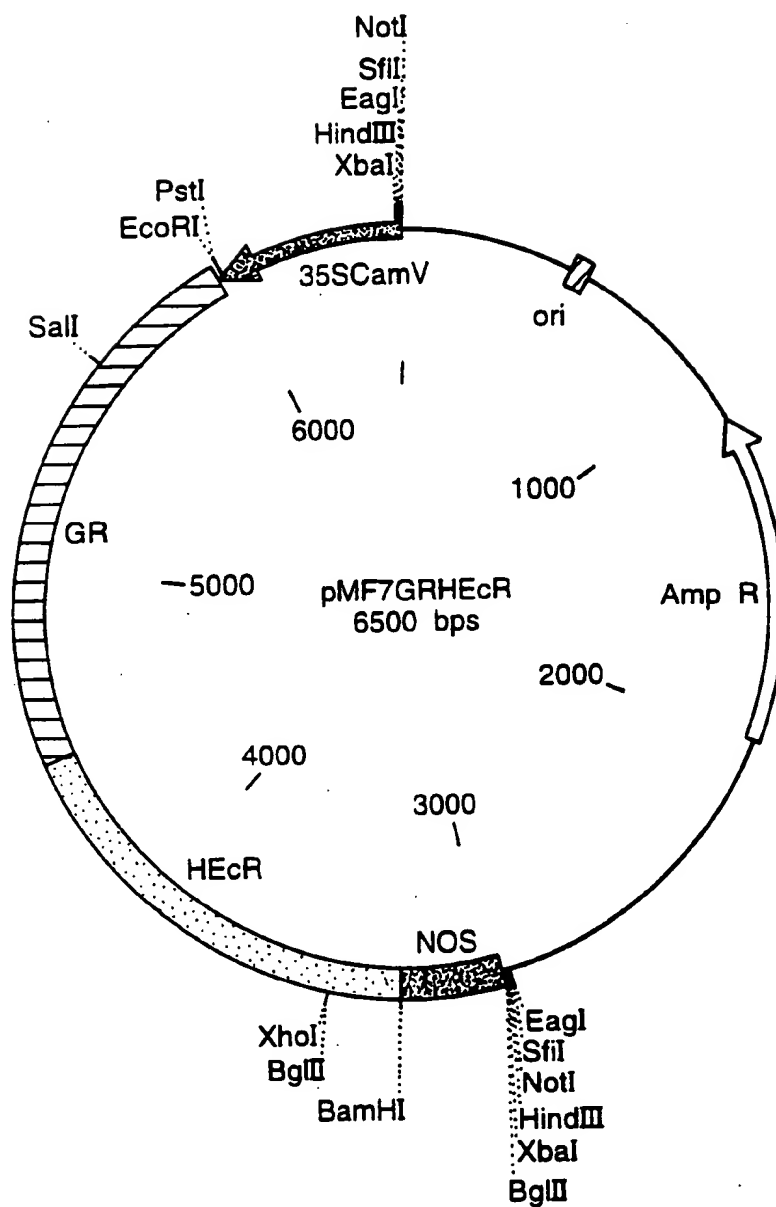
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Fig.22.



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Fig.23.



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Fig.24.

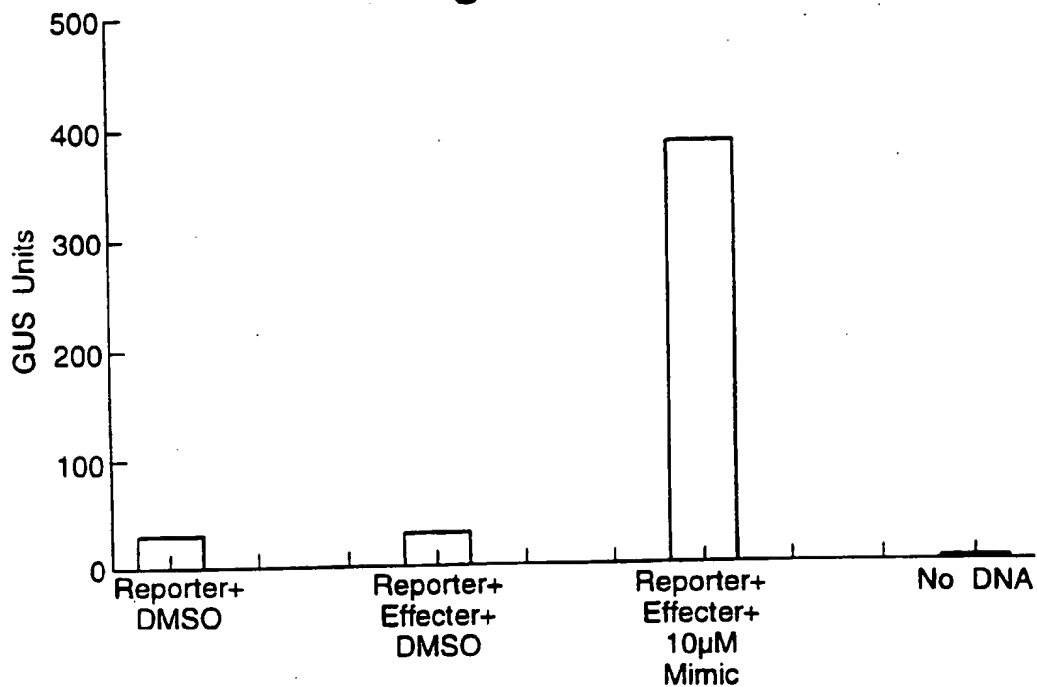
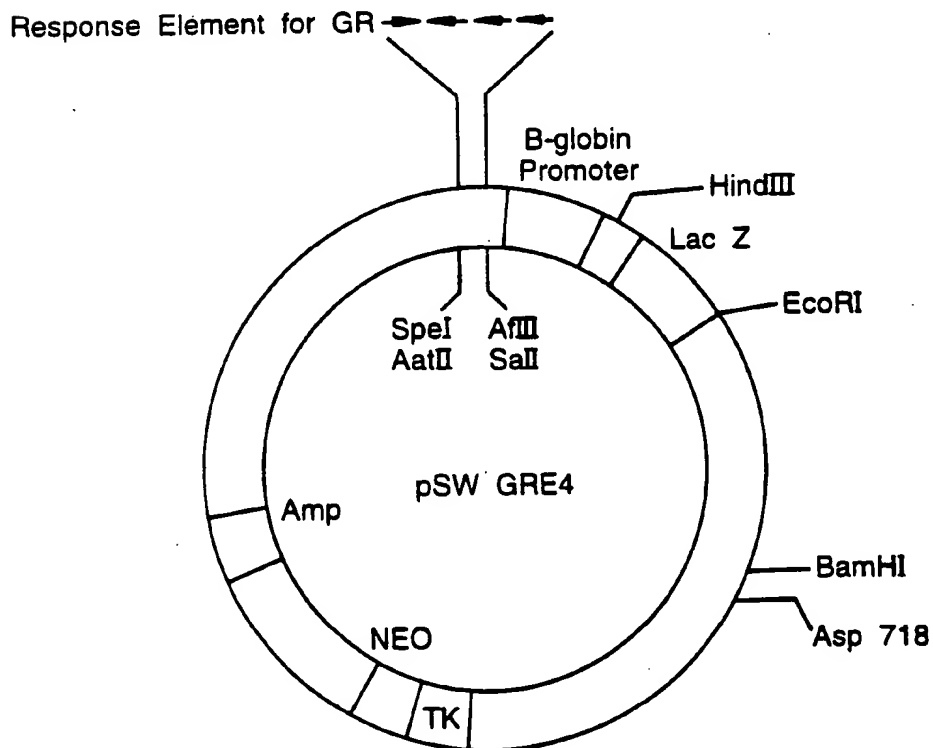
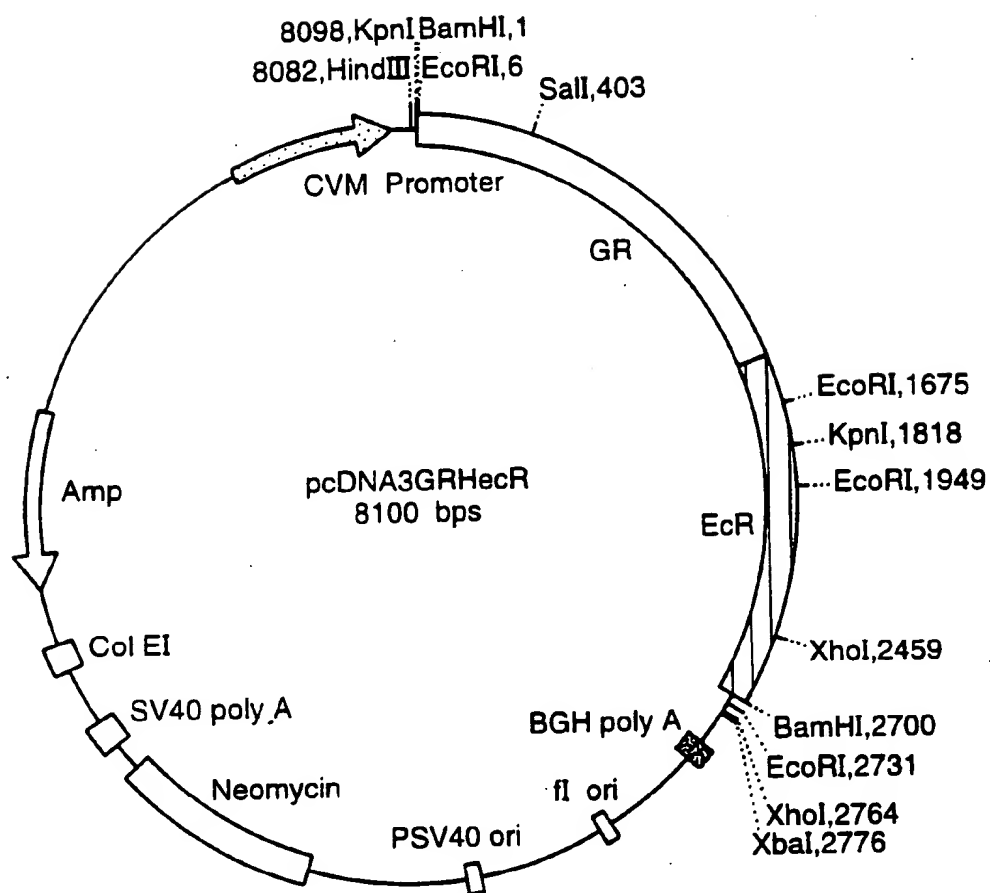


Fig.26.



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Fig.25.



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Fig.27.

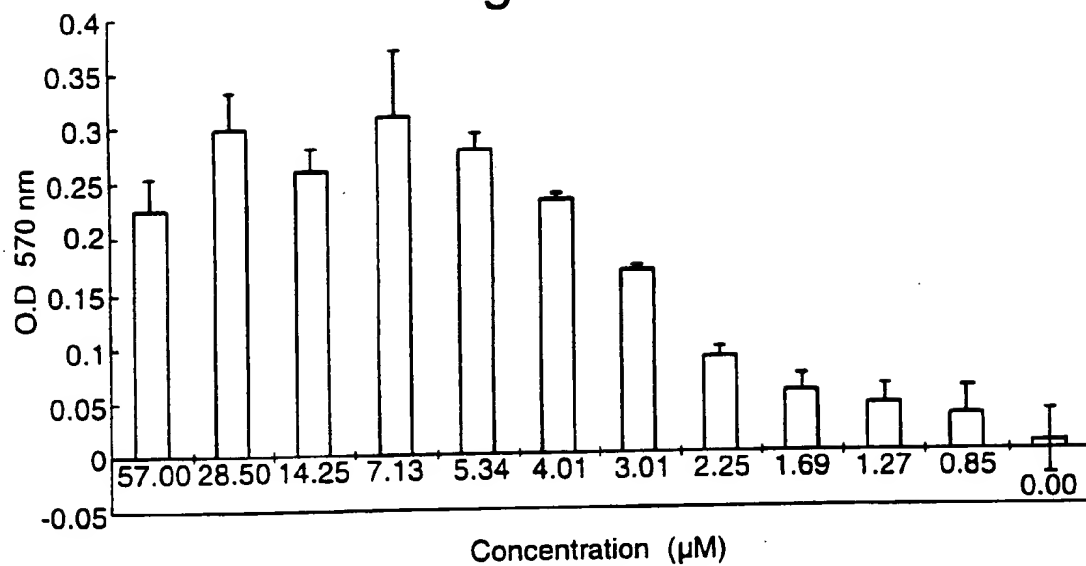
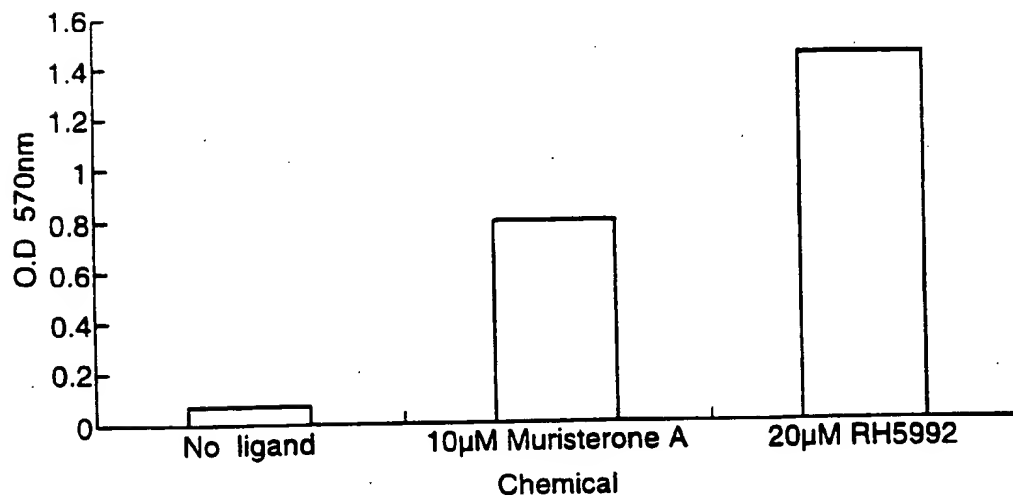


Fig.28.



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Fig.29.

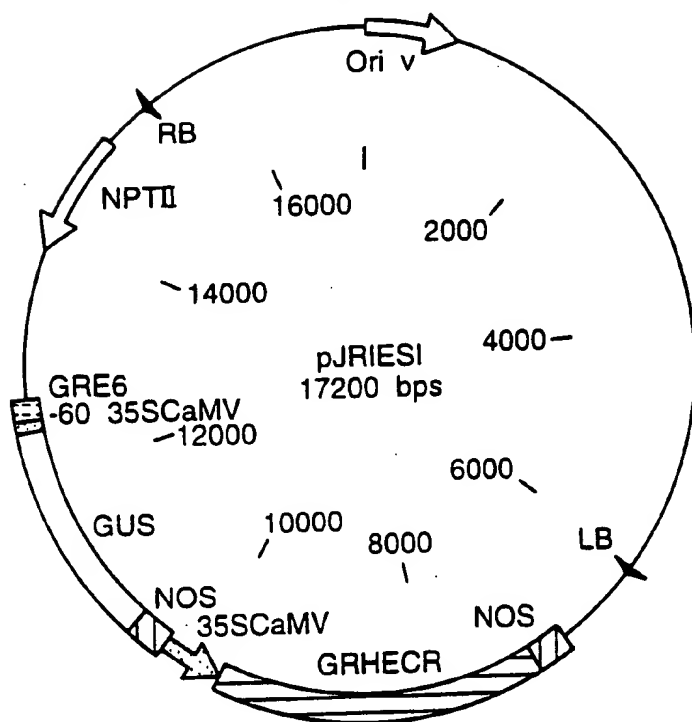
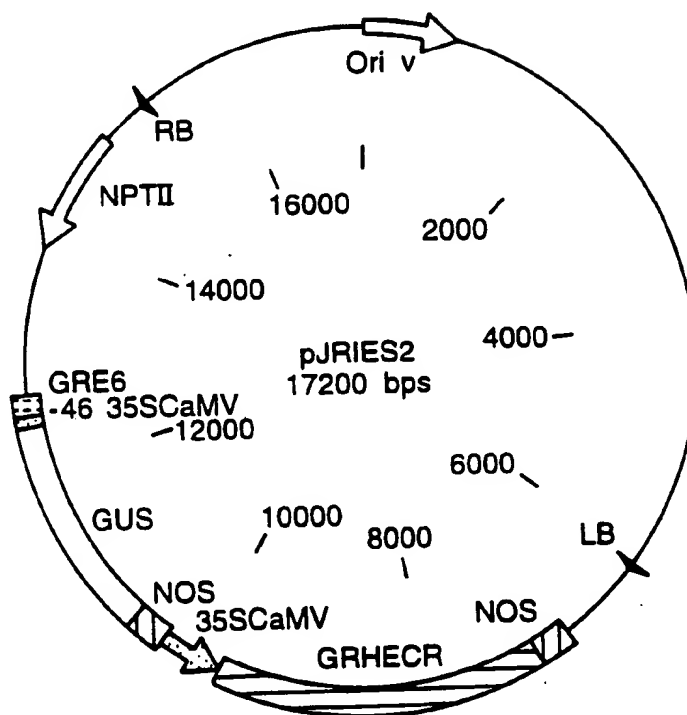


Fig.30.



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Fig.31.

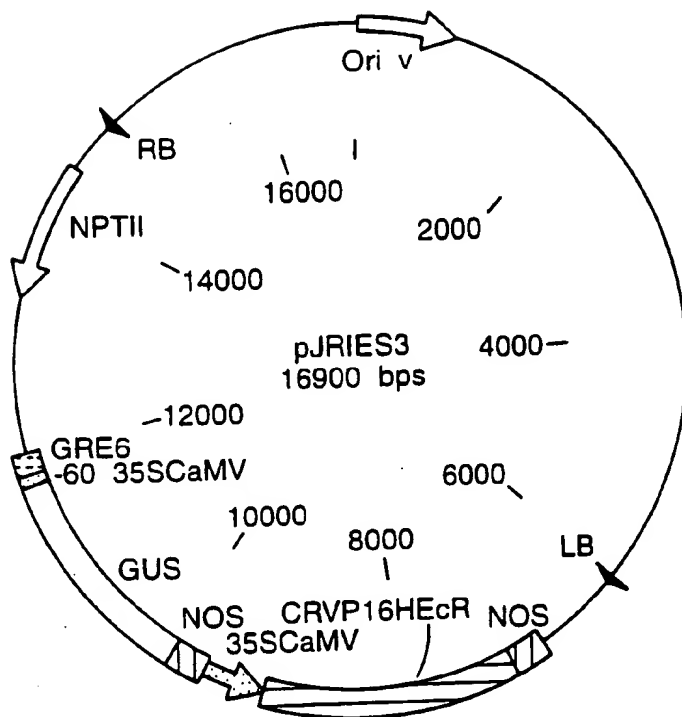


Fig.32.

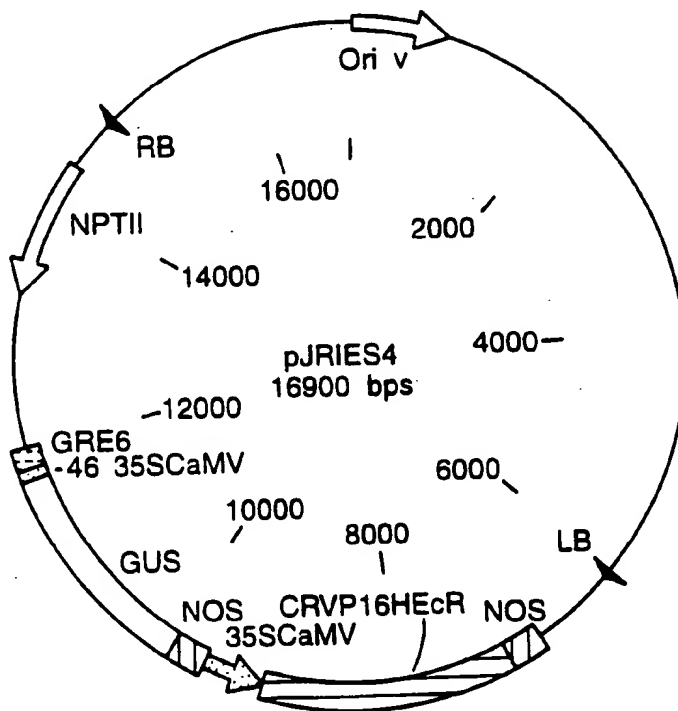


Fig.33.

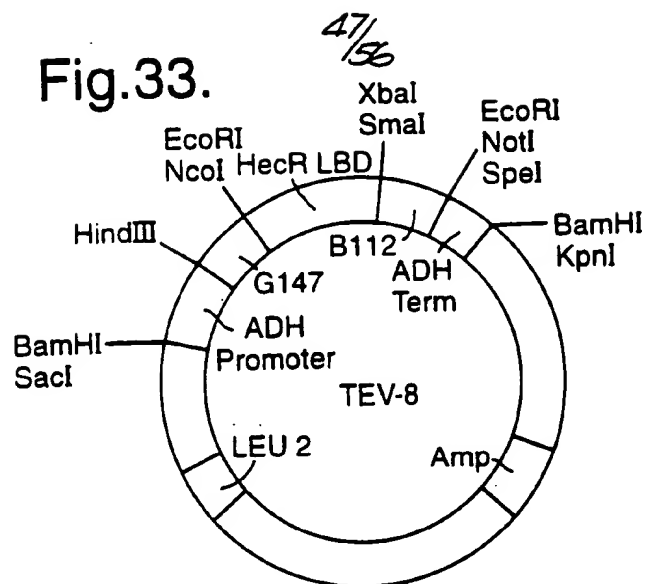


Fig.34.

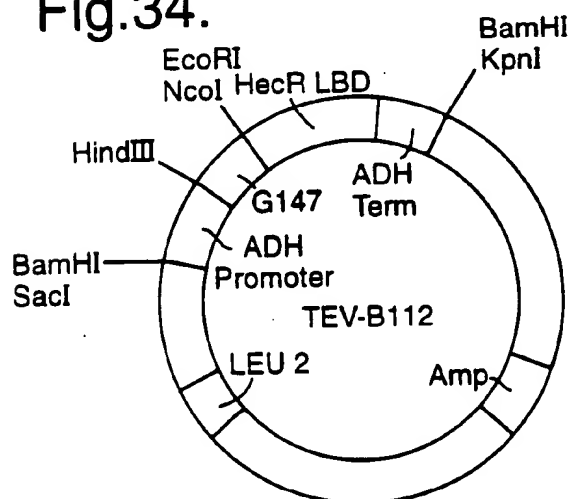
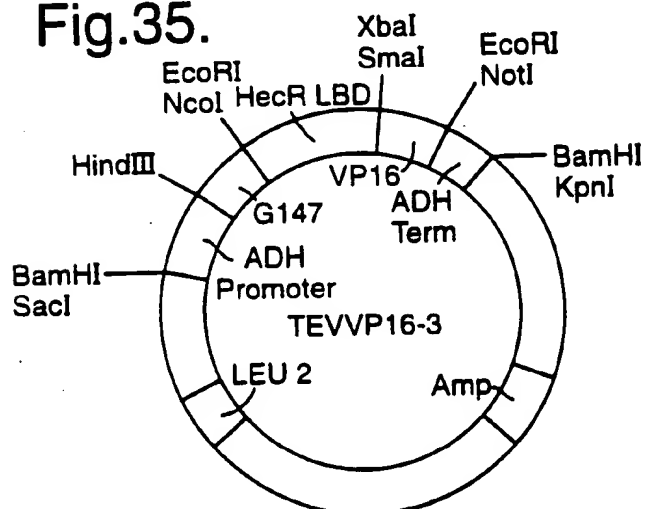
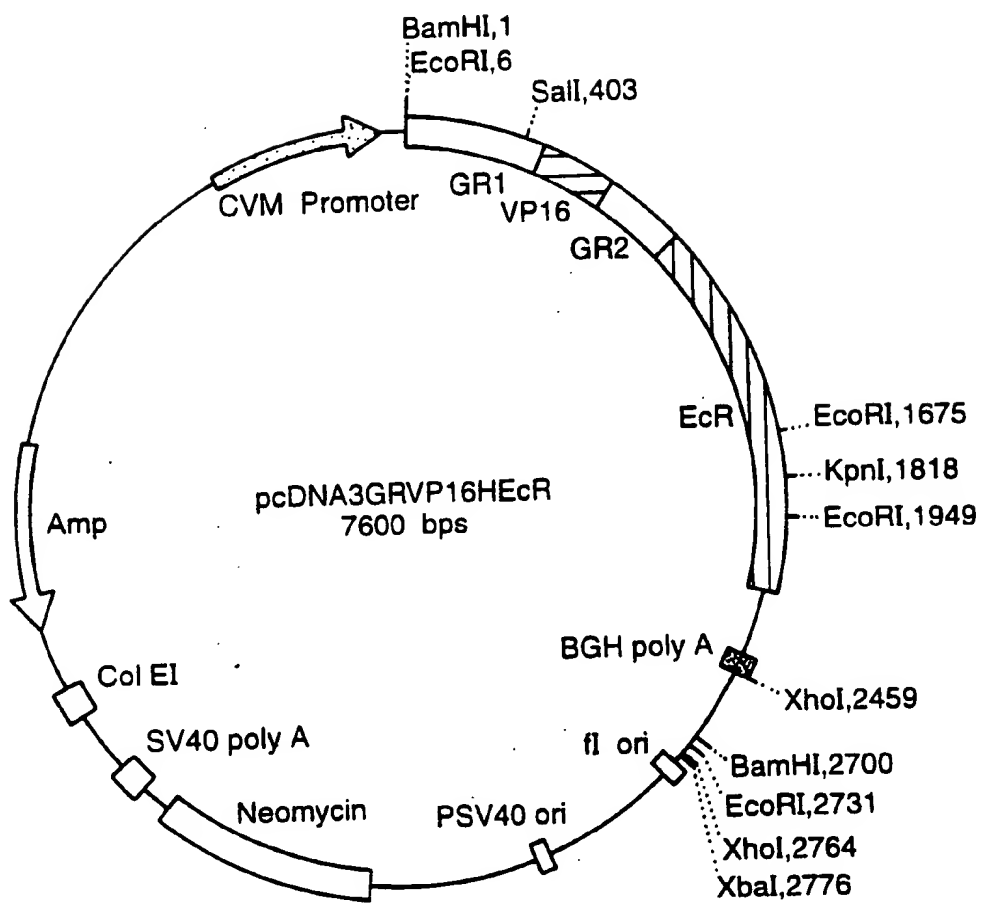


Fig.35.



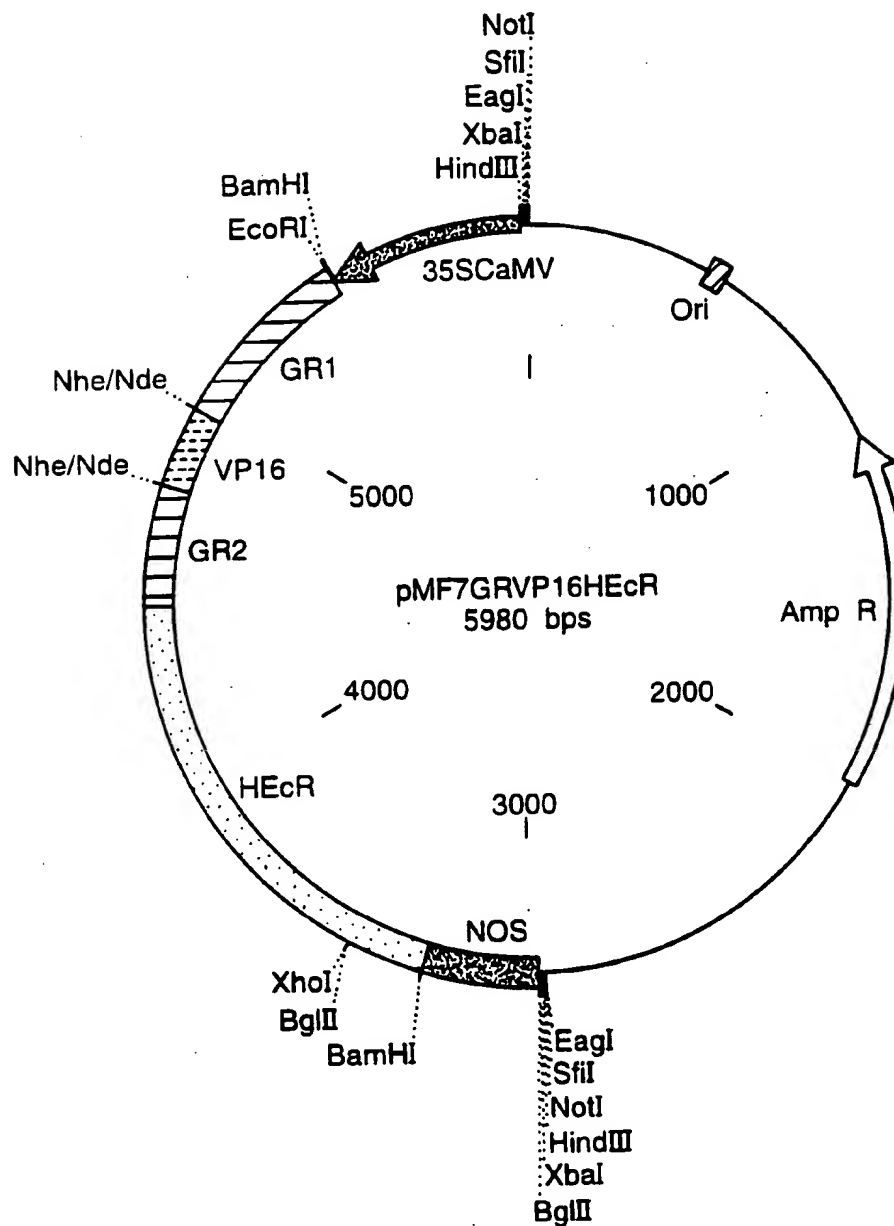
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Fig.36.



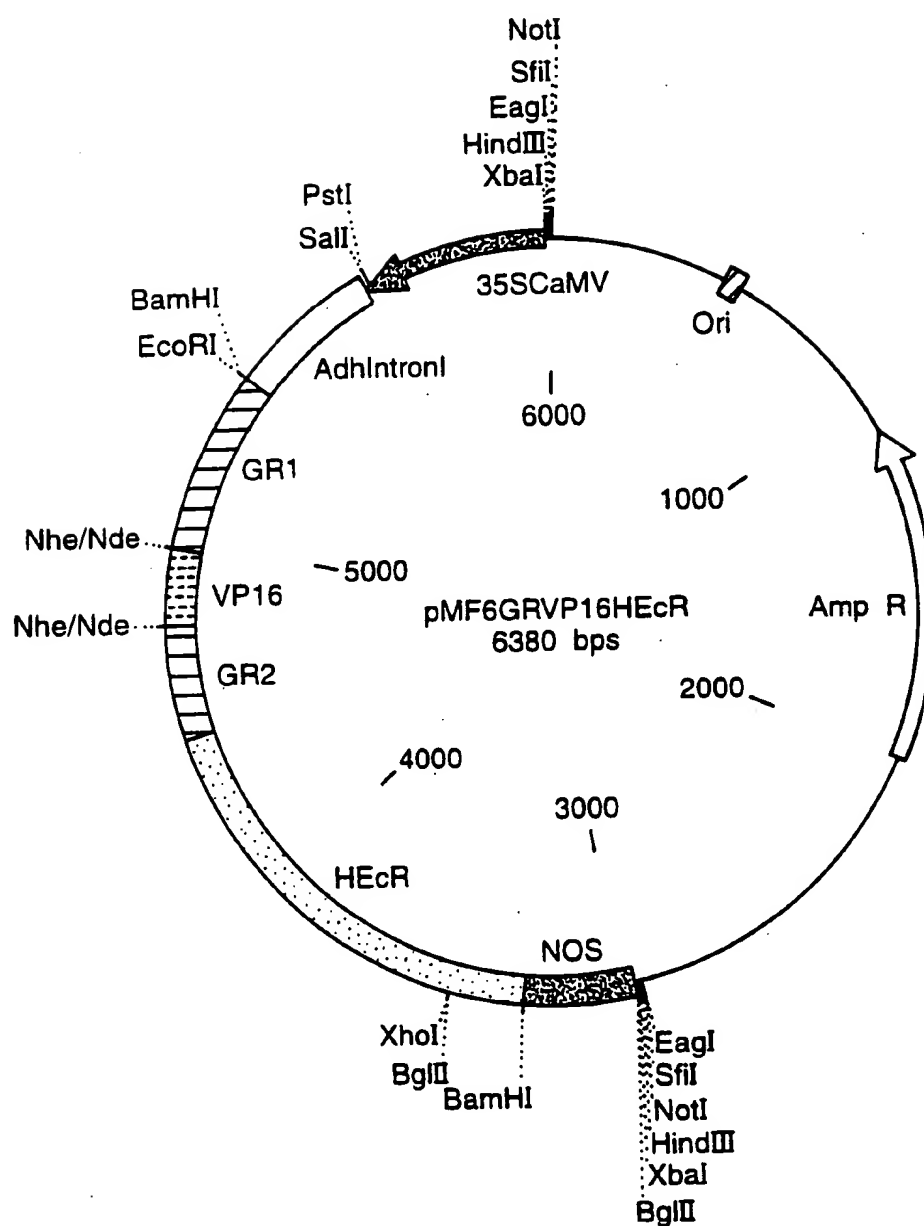
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Fig.37.

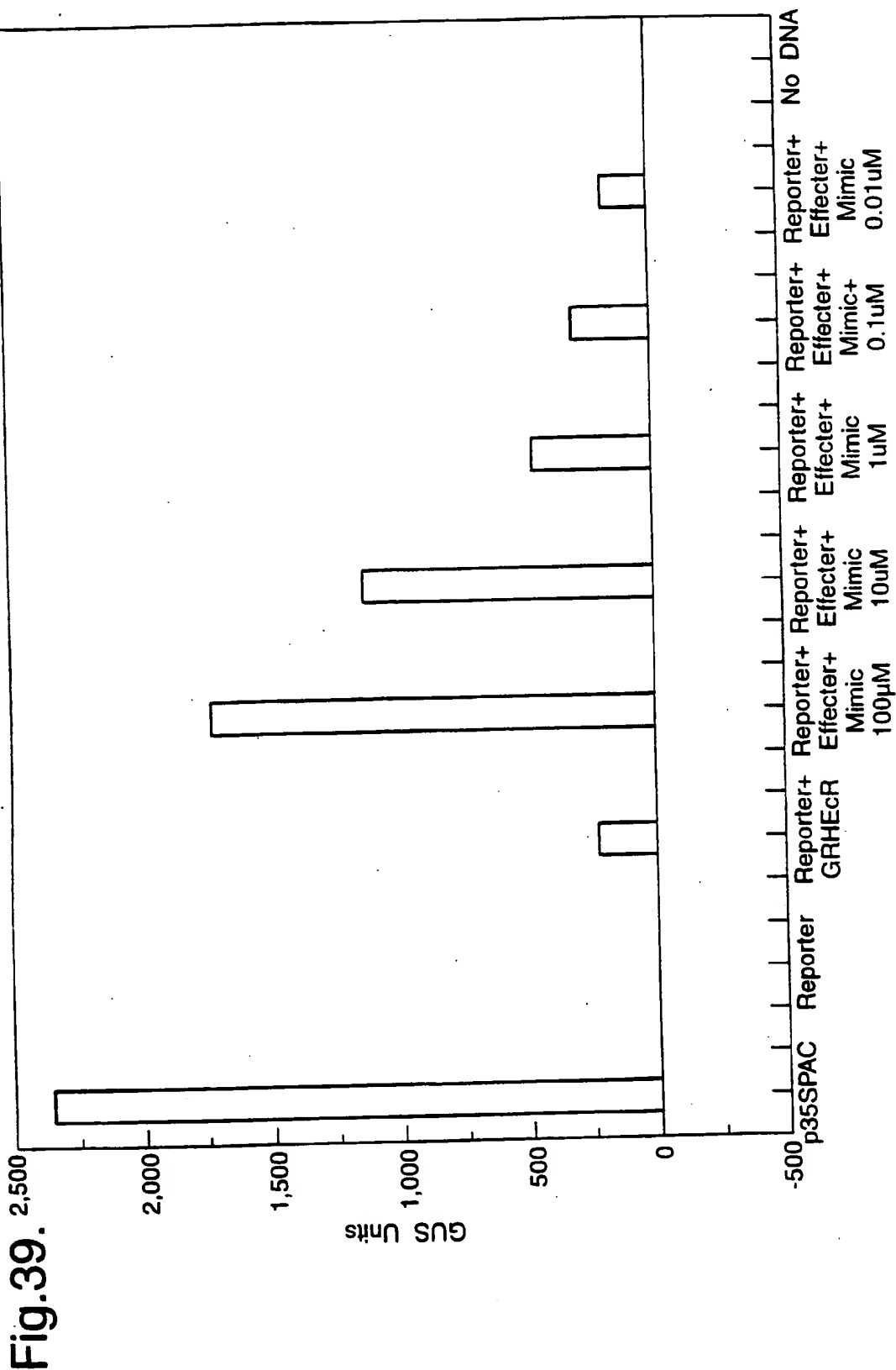


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Fig.38.



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Spodoptera exigua DNA sequence.

Fig.40.

Sequence ID 6

SPODOPTERA EXIGUA HINGE AND LIGAND BINDING DOMAINS

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1	AGG CCG GAG TGC GTG GTG CCA GAA AAC AAC CAG TGT GCA ATG AAA AGG	3	9	15	21	27	33	39	45
	TCC GGC CTC ACG CAC CAC GGT CTT TTG GTC ACA CGT TAC TTT TCC								
46	AAA GAG AAA AAG GCA CAA AGG GAA AAA GAC AAG TTG CCA GTC AGT								
	TTT CTC TTT TTC CGT GTT TCC CTT TTT CTG TTC AAC GGT CAG TCA								
91	ACA ACG ACA GTG GAT GAT CAC ATG CCT CCC ATT ATG CAG TGT GAT								
	TGT TGC TGT CAC CTA CTA GTG TAC GGA GGG TAA TAC GTC ACA CTA								
136	CCA CCG CCT CCA GAG GCC GCA AGA ATT CAC GAG GTG CCA CGA								
	GGT GGC GGA GGT CTC CGG CGT TCT TAA GTG CTC CAC GGT GCT								
181	TTT CTC AAT GAA AAG CTA ATG GAC AGG ACA AGG CTC AAG AAT GTG								
	AAG GAC TTA CTT TTC GAT TAC CTG TCC TGT TCC GAG TTC TTA CAC								
226	CCC CCT CAC TGC CAA CCA GAA GTC CTT AAT AGC GAG GCT GGT CTG								
	GGG GGA GTG ACG GTT GGT CTT CAG GAA TTA TCG CTC CGA CCA GAC								
271	GTA CCA AGA AGG CTA TGA ACA GCC ATC AGA GGA TCT AAA AAG								
	CAT GGT TCT TCC GAT ACT TGT CGG TAG TCT TCT CCT AGA TTT TTC								

Fig.40 i.

316 AGT CAC ACA GTC GGA TGA AGA CGA AGA AGA GTC GGA CAT GCC GTT
 TCA GTG TGT CAG CCT ACT TCT TCT TCT CAG CCT GTA CGG CAA
 361 CCG TCA GAT CAC CGA GAT GAC GAT CCT CAC AGT GCA GCT CAT TGT
 GGC AGT CTA GTG GCT CTA CTG CTA GGA GTG TCA CGT CGA GTA ACA
 406 TGA ATT CGC TAA GGG CCT ACC AGC GTT CGC AAA GAT CTC ACA GTC
 ACT TAA GCG ATT CCC GGA TGG TCG CAA GCG TTT CTA GAG TGT CAG
 451 GGA TCA GAT CAC ATT ATT AAA GGC CTG TTC GAG TGA GGT GAT GAT
 CCT AGT CTA GTG TAA TAA TTT CCG GAC AAG CTC ACT CCA CTA CTA
 496 GTT GCG AGT AGC TCG GCG GTA CGA CGC GGC GAC AGA CAG CGT GTT
 CAA CGC TCA TCG AGC CGC CAT GCT GCG CGG CTG TCT GTC GCA CAA
 541 GTT CGC CAA CAA CCA GGC GTA CAC CCG CGA CAA CTA CCG CAA GGC
 CAA GCG GTT GTT GGT CCG CAT GTG GGC GCT GTT GAT GGC GTT CCG
 586 AGG CAT GGC CTA CGT CAT CGA GGA CCT GCT GCA CTT CTG CCG GTG
 TCC GTA CCG GAT GCA GTA GCT CCT GGA CGA CGT GAA GAC GGC CAC
 631 CAT GTA CTC CAT GAT GAT GGA TAA CGT CCA CTA TGC ACT GCT CAC
 GTA CAT GAG GTA CTA CTA CCT ATT GCA GGT GAT ACG TGA CGA GTG
 676 TGC CAT CGT CAT TTT CTC AGA CCG ACC CCG GCT TGA GCT AAC CCT
 ACG GTA GCA GTA AAA GAG TCT GGC TGG GCC CGA ACT CGA TTG GGA
 721 GTT GGT GGA GGA GAT CCA GAG ATA TTA CCT GAA CAC GCT GCG GGT
 CAA CCA CCT CCT CTA GGT CTC TAT AAT GGA CTT GTG CGA CGC CCA

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Fig.40 ii. 766 GTA CAT CCT GAA CCA GAA CAG TCG GTC GCC GTG CTG CCC TGT CAT
 CAT GTA GGA CTT GGT CTT GTC AGC CAG CGG CAC GAC GAG GGG ACA GTA

811 CTA CGC TAA GAT CCT CGG CAT CCT GAC GGA GCT GCG GAC CCT GGG
 GAT GCG ATT CTA GGA GCC GTA GGA CTG CCT CGA CGC CTG GGA CCC

856 CAT GCA GAA CTC CAA CAT GTG CAT CTC ACT ACT GAA GAA CAG
 GTA CGT CTT GAG GTT GTA CAC GTA GAG TGA GTT CGA CTT CTT GTC

901 GAA CGT GCC GCC GTT CTT CGA GGA TAT CTG GGA CGT CCT CGA GTA
 CTT GCA CGG CGG CAA GAA GCT CCT ATA GAC CCT GCA GGA GCT CAT

946 AAA
 TTT

Total number of bases is: 948.

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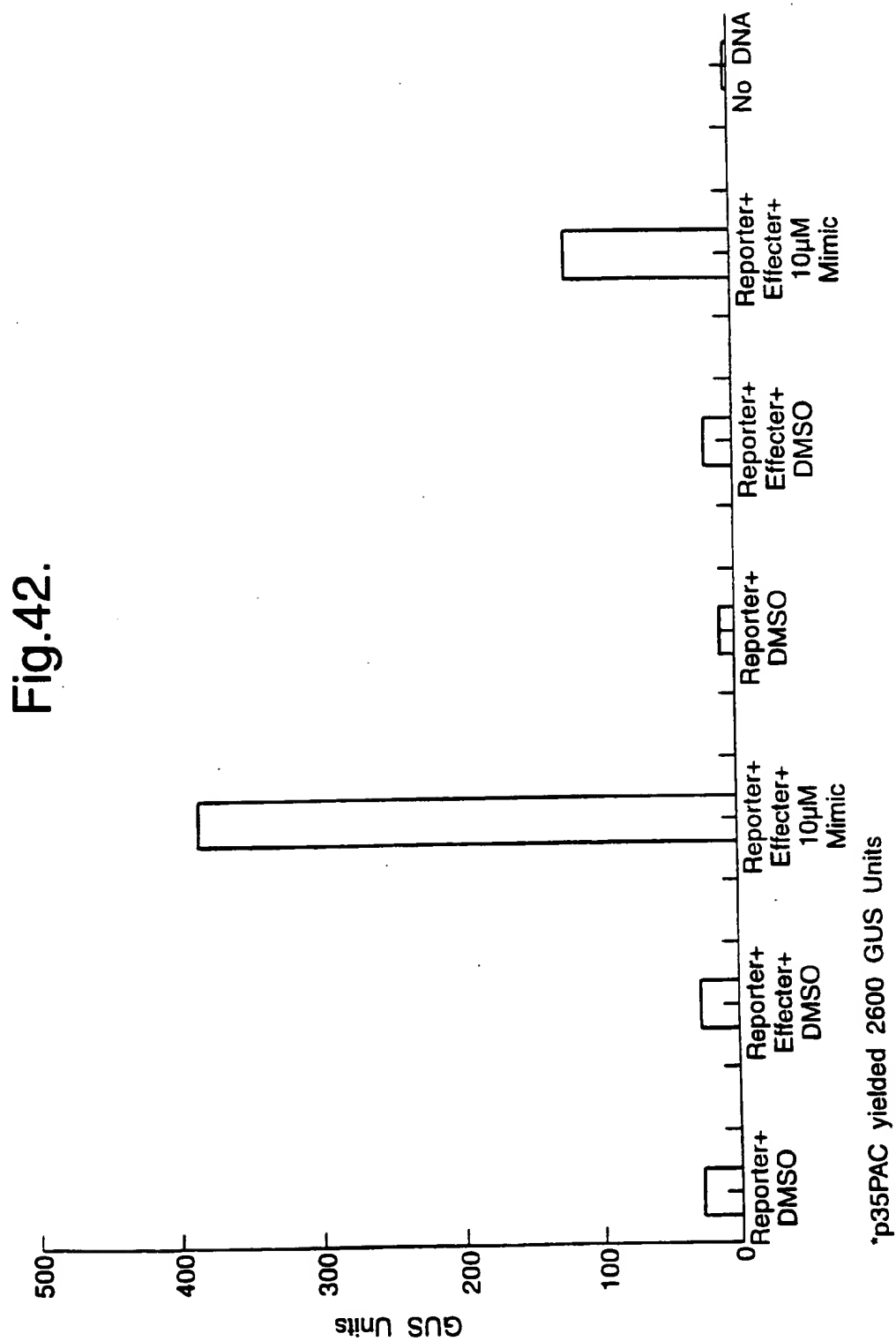
Fig.41.

Sequence I.D. 7

Sequence comparison between Heliothis 19R clone and SECR Taq clone

HECR	RPECVVPENQCAMKRKEKKAQREKDKLPVSTTTVDDHMPPIMQCDPPPEAARILECVQ
SECR	RPECVVPENQCAMKRKEKKAQREKDKLPVSTTTVDDHMPPIMQCDPPPEAARI
HECR	HEVVPFRFLNEKLMQNRLKNVPPLTANQKSLIARLVWYQEGYEQPSEEDLKRVQTQSD
SECR	HEVVPFRFLNEKLMERTRLRNVPPLTANQKSLIARLVWYQEGYEQPSEEDLKRVQTQSD
HECR	EDDESDMPFRQIT EMT ILTVQLIVEFAKGLPGFAKISQSDQITLLKACSSSEVMMLR
SECR	EDEESDMPFRQIT EMT ILTVQLIVEFAKGLP AF AKISQSDQITLLKACSSSEVMMLR
HECR	VARRYDAA T DSVLFANNQAYTRDNYRKAGMAYVIEDLLHFCRCMYSMMMDNVHYALL
SECR	VARRYDAA T DSVLFANNQAYTRDNYRKAGMAYVIEDLLHFCRCMYSMMMDNVHYALL
HECR	TAIVIFSDRPGLEOPL L VEEIQRYYLNTLRVYILNQNSASPRGAVIFGEILGILTEI
SECR	TAIVIFSDRPGLELT L VEEIQRYYLNTLRVYILNQNSRSPCCPVIYAKILGILTEL
HECR	RTLGMQNSNMCI S LK L KKRKLPPFLEEIDWDV
SECR	RTLGMQNSNMCI S LK L KNRNVPFF E IDWDV

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 96/01195

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C12N15/85 C12N15/62 C07K14/72 C07K19/00
C12N5/10 A61K38/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 03162 (GENENTECH INC) 18 February 1993	4,5,44, 92-99
Y	see abstract; claims 1-27; figure 1	1,3, 8-43, 45-49, 51-91
X	WO,A,91 13167 (UNIV LELAND STANFORD JUNIOR) 5 September 1991	4,5,44, 50,93-99
Y	see abstract; claims 2,24	2,3
	-/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

9 August 1996

Date of mailing of the international search report

19. 08. 96

Name and mailing address of the ISA

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INTERNATIONAL SEARCH REPORT

 Int. Patent Application No.
 PCT/GB 96/01195

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CELL, OCT 4 1991, 67 (1) P59-77, UNITED STATES, XP002010069 KOELLE MR ET AL: "The Drosophila EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily."	4,5
Y	see the whole document	1-3, 8-43, 45-49, 51-92
X	--- INSECT BIOCHEM MOL BIOL, JAN 1993, 23 (1) P115-24, ENGLAND, XP002010070 IMHOF MO ET AL: "Cloning of a Chironomus tentans cDNA encoding a protein (cEcRH) homologous to the Drosophila melanogaster ecdysteroid receptor (dEcR)." see the whole document	4,5
X	--- INSECT BIOCHEM MOL BIOL, JAN 1995, 25 (1) P19-27, ENGLAND, XP002010071 CHO WL ET AL: "Mosquito ecdysteroid receptor: analysis of the cDNA and expression during vitellogenesis." see the whole document	4,5,52, 53
Y	--- EP,A,0 615 976 (AMERICAN CYANAMID CO) 21 September 1994 see page 6, line 28 - line 32; claims 1-12; example 2	8-43, 45-49, 51-92
Y	--- EUR. J. ENTOMOL. (1995), 92(1), 333-40 CODEN: EJENE2;ISSN: 1210-5759, XP002010346 SMAGGHE, GUY ET AL: "Biological activity and receptor -binding of ecdysteroids and the ecdysteroid agonists RH-5849 and RH-5992 in imaginal wing discs of Spodoptera exigua (Lepidoptera: Noctuidae)" see page 336, paragraph 3 - page 337, paragraph 2	51-65
A	--- DEVELOPMENTAL GENETICS, 1995, 17, 319-330, XP002010345 KOTHAPALLI R ET AL: "CLONING AND DEVELOPMENTAL EXPRESSION OF THE ECDYSONE RECEPTOR GENE FROM THE SPRUCE BUDWORM, CHORISTONEURA-FUMIFERANA" see the whole document --- -/-	1-5, 51-54

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 96/01195

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	INSECT BIOCHEM. MOL. BIOL. (1994), 24(8), 763-73 CODEN: IBMBES;ISSN: 0965-1748, XP002010072 JINDRA, MAREK ET AL: "Isolation and developmental expression of the ecdysteroid-induced GHR3 gene of the wax moth <i>Galleria mellonella</i> " see the whole document ---	1-5
A	US,A,5 424 333 (WING KEITH D) 13 June 1995 see column 150, paragraph 3 - paragraph 7; example 3 -----	97,98

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 96/01195

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INTERNATIONAL SEARCH REPORT

International application No.

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Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 98
because they relate to subject matter not required to be searched by this Authority, namely:
Although this claim is directed partly to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/GB 96/01195

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		AU-B- 602505	18-10-90
		AU-B- 6926687	03-09-87
		CA-A- 1295618	11-02-92
		EP-A- 0234944	02-09-87
		ES-T- 2032818	16-07-96
		KR-B- 9410277	22-10-94
		AU-B- 599970	02-08-90
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